THE EFFECT OF GLUTAMINE ON THE HEAT SHOCK PROTEIN CONTENT OF MUSCLE IN CELL CULTURE AND DURING CRITICAL ILLNESS

Thesis submitted in accordance with the requirements of the University of

Liverpool for the degree of Doctor of Medicine by Thomas Aloys Bongers

May 2012

UNIVERSITY OF LIVERPOOL

Abstract

Despite significant developments, critical care mortality remains high at ~ 20-30%. Muscle wasting with protein breakdown is frequently seen in critically ill patients with increased amino acids release from muscle tissue, of which alanine and glutamine compose a large proportion. Plasma glutamine is rapidly utilized and circulating plasma glutamine declines rapidly. Low plasma glutamine concentrations correlate with mortality in critical illness and intravenous glutamine supplementation improves survival of the critically ill patient. The precise mechanism whereby this protection is afforded remains uncertain, although evidence suggests that glutamine plays an important role in the ability of cells to respond to stress.

The expression of stress or heat shock proteins (HSPs) is one of the most highly conserved mechanisms of cellular protection. Increased intracellular HSP content is associated with a striking preservation of muscle mass and function and low muscle and serum HSP 70 content in severe trauma correlate with increased mortality. Glutamine infusions facilitate increased HSP expression. Further, glutamine infusions enhance HSP content in multiple organs of the rat with a significant protection of these organs from damage during sepsis and a mortality advantage. This has resulted in the hypothesis that glutamine deficiency leads to a modified HSP content of muscle cells and a diminished ability to mount a stress response. Administration of glutamine will have a direct and beneficial effect on the HSP content of muscle in cell culture and during critical illness.

To test the hypothesis a muscle cell culture model was established and cells treated with a range of glutamine concentrations during differentiation (0.1-10mM glutamine). The effect of modified glutamine concentration on the ability of cells to mount a stress response to hyperthermia and TNF-α stress (0.5-5mM glutamine) was determined. A pilot clinical trial (which was double blind randomized controlled) was also undertaken which comprised of treatment of patients with sepsis with one large intravenous glutamine dose (0.5g/kg bodyweight) early during critical illness. Muscle HSP content was measured at baseline and at 48 and 96 hours after initiation of treatment.

During myoblast cell differentiation, an extracellular glutamine deficiency correlated with increased HSP 70 content; high extracellular glutamine concentration resulted in little additional benefit. During stress (either hyperthermia or TNF-α treatment) extracellular glutamine deficiency correlated with a reduced intracellular HSP 70 (highly inducible) content following stress compared with cells grown in normal extracellular glutamine concentrations. Glutamine intervention in human subjects demonstrated a significant correlation between plasma glutamine and muscle content of HSP 10, α B crystallin and HSC 70 (constitutively expressed).

Results emphasise the importance to correct a glutamine deficiency during stress and the need to maintain normal plasma glutamine concentrations during recovery, facilitating muscle regeneration and repair. A correlation was observed between plasma glutamine concentrations and muscle HSPs. However, one single dose of glutamine did not change the plasma glutamine concentration significantly and can therefore not be advocated.

ACKNOWLEDGEMENTS

I am indebted to my patients and their families who agreed to participate in the clinical trial at a very difficult and stressful time of their life.

I thank my supervisors Professor Anne McArdle and Professor Richard Griffiths, who inspired me to undertake this work. Their advice and encouragement proved to be invaluable throughout. I thank my colleagues in the department of Musculoskeletal Biology in the Institute of Ageing and Chronic Disease for their help, advice and support. I specifically want to thank Dr Claire Routley; without her help I would have not been able to present this work now. I am very grateful to Dr Anna Kayani Dr Adam Lightfoot and Dr Aphrodite Vasilaki for their help and support.

I thank the nursing staff of the Critical Care Unit at Whiston Hospital and at the Critical Care Unit of the Royal University Hospital, Liverpool for administering the trial solutions to patients and for identifying potential patients. I thank Mr Rob Fisher for preparing trial solutions and performing trial randomization at the Pharmacy Department at Whiston Hospital.

I thank Fresenius Kabi for their generous financial support towards the cost of the clinical trial and for supplying the alanine-glutamine dipeptide for the clinical trial. Fresenius Kabi was not involved in the clinical trial other than financial support, did not participate in the analysis of the data or influence the results in any way. I am indebted to the Intensive Care Society for providing a research grant for the clinical trial (Young Investigators Award).

I am grateful to my wife and my four children Ida, Clara, Maja and Liliana for their support, encouragement to do this work and their endless patience throughout the completion of this work.

STATEMENT OF WORK DONE BY THE AUTHOR AND OTHERS

All work described in the thesis was performed by the author, except the following:

- Measurement of the C-reactive protein was performed by the Biochemistry department of the Royal Liverpool University Hospital or the Biochemistry department of Whiston Hospital.

- Measurement of the White Cell count was performed by the Haematology department of the Royal Liverpool University Hospital or the Haematology department of Whiston Hospital.

- Mr Rob Fisher and the Pharmacy aseptic unit at Whiston Hospital performed the Randomisation

- The Pharmacy aseptic unit at Whiston Hospital and the Pharmacy aseptic unit at Royal Liverpool University Hospital performed the preparation of the trial drugs (glutamine and placebo).

- Physiological measurements for example temperature, blood pressure etc. were performed by the nursing staff of the critical care units of Whiston Hospital and the Royal Liverpool University Hospital as part of the routine care of critically ill patients.

- Measurements of the plasma glutamine concentration was performed by High Performance Liquid Chromatography (HPLC) at the MRC biochemistry research group at Oxford University.

Dedicated to my mother and my brother Hans-Werner,

who always encouraged my ambitions.

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ABBREVIATIONS

Ala-Gln	Alanin-glutamine
ALT	Alanin amino transferase
AMP	Adenosine monophosphate
APS	Ammonium persulphate
APACHE II	Acute Physiology and Chronic Health Evaluation II
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CI	Confidence interval
CRP	C-reactive protein
DC	Dendritic cells
DMEM	Dulbecco's modified Eagle medium
DoH	Department of Health
D-PBS	Dulbecco's- phosphate buffered saline
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
ELISA	Enzyme Linked Immuno Sorbent Assay
FCS	Foetal calf serum
GABA	gamma-amminobutyric acid
HDU	High dependency unit
HSE	Heat Shock Element
HSF	Heat Shock Factor
HSP	Heat Shock Protein
ICNARC	Intensive Care National Audit and Research Centre
ICU	Intensive care unit
IKK	Inhibitor kappa B protein kinase
IL 1β	Interleukin 1beta
IL 6	Interleukin 6
IL 10	Interleukin 10
INR	International normalized ratio
Iv	Intravenous
LPS	Lipopolysaccharide
MHRA	Medicines and Healthcare products regulatory agency

MOF	Multi organ failure
NADPH	Nicotinamide adenine dinucleotide phosphate
NF kappa B	Nuclear factor kappa B
NK	Natural killer cells
NO	Nitric Oxide
PBS	Phosphate buffered saline
PMNC	Polymorphonuclear cells
PMSF	Phenylmethylsulphonyl fluoride
PN	Parenteral nutrition
RCT	Randomised controlled trial
ROS	Reactive oxygen species
RR	Risk ratio
SAPS	Simplified Acute Physiology Score
SDS	Sodium dodecyl sulphate
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential Organ Failure Assessment
SSA	Sulphosalicylic acid
TCA cycle	Tricarboxylic acid cycle
TEA	Triethanolamine
TEMED	NNN'N'-tetramethylethylene-diamine
TNF-α	Tumour Necrosis Factor-α
TPN	Total parenteral nutrition
WCC	White cell count

Chapter 1

Introduction

1.1. Critical illness - Magnitude of problem

1.1.1. Critical care medicine – Background

Critical care medicine has developed greatly in the last 50 years. This development was initiated by the polio epidemic in the early 1950s when there was insufficient respiratory support available for polio sufferers. This led to the use of life support techniques usually employed in operating theatres. Further, constant attendance of medical staff and establishing designated areas lead to a significant mortality reduction. Increasingly complex interventions have been made feasible by the ability to replace the role of a failing organ until recovery occurs. Critical care has developed greatly, for example more sophisticated ventilation techniques, cardiovascular support and renal support are now routinely employed. This development was necessitated by increasing demand and development in medicine and surgery.
Different to other specialities, the decision to admit to critical care is based not on the nature of the disease suffered but by the support needed by the individual patient. This is illustrated by the definition of level of care published by the Department of Health. (DoH 2000) which stipulates the need of critical care i.e. admission to a High Dependency Unit (Level 2) by meeting the following criteria:
"Patients requiring more detailed observation or intervention including support for a single failing organ system or post-operative care and those 'stepping down' from higher levels of care."
The document further stipulates the need of critical care i.e. admission to an Intensive Care Unit (Level 3) by meeting the following criteria:
"Patients requiring advanced respiratory support alone or basic respiratory support together with support of at least two organ systems. This level includes all complex patients requiring support for multi-organ failure."
This definition of critical care based on clinical features rather than different predisposing conditions create a rather heterogeneous critical care population (Carson and Shorr. 2003). The ultimate outcome will be influenced at least in part by the predisposing condition as well as the clinical syndrome the patients present with.

The Systemic inflammatory response syndrome (SIRS) represents a non-specific inflammatory response following a variety of severe clinical insults. The term 'systemic inflammatory response syndrome' (SIRS) was proposed by the consensus conference of experts in 1991 for the description of a non-specific inflammatory process (Bone et al. 1992).

SIRS represents the physiological derangements in these conditions.

SIRS is defined by the presents of two or more of the following criteria:

(1) Temperature >38 or <36°C;

(2) Heart rate >90 beats per minute;

(3) Respiratory rate >20 breaths per minute or pCO <32 mm Hg;

(4) White blood cell count >12 x 10^9/l or <4 x 10^9/l.

Following this definition it can be safely assumed that almost every patient admitted to critical care in the UK fulfils the definition of SIRS (Harrison et al. 2004). These insults can be as divergent as surgery, trauma, burns, infections, pancreatitis and other diseases (Claus et al. 2010).

Regardless of its aetiology, any insult, if severe enough, induces the release of pro-inflammatory mediators and the formation of reactive oxygen intermediates, likely as a consequence of activation of polymorphonuclear leukocyte and other reticule-endothelial cells (Brown et al. 2006; Gutteridge and Mitchell. 1999; Marshall. 2005).

Many attempts have been made to quantify the degree of severity of illness for patients admitted to critical care. And indeed it has been shown that the severity of acute illness can be quantified by the degree of disturbance of a broad range of physiologic variables (Rowan et al.1994). Several scoring systems were developed to aid quantitative assessment of the severity of illness, stratification of patient groups and evaluation of mortality within a critical care unit and between critical care units (Peter and Welte. 1997; Unertl and Kottler. 1997). Scoring systems were introduced due to the heterogeneity of critical care patients to objectively assess severity of illness. This enabled prognostication based on risk estimates derived from prognostic systems for the interpretation of crude mortality rates, potentially allowing inter-hospital and international comparison of clinical performance and quality of care (Carson S, 2003; Peter and Welte. 1997; Unertl and Kottler. 1997).

The Acute Physiology and Chronic Health Evaluation II (APACHE II) score (Knaus et al. 1985) is one such score commonly used in UK critical care units (Harrison et al. 2004; Rowan et al. 1994).

The APACHE II score is validated for the use in the UK assessing the following 12 parameters (Knaus et al. 1985): temperature, mean arterial pressure, heart rate, respiratory rate, the alveolar–arterial gradient, arterial pH, serum sodium content, serum potassium content, serum creatinine content, haematocrit, white cell count, Glasgow coma scale. Points are added for age above 44 years on a graded scale. Points are further added for the presents of chronic disease states in combination with the mode of presentation, i.e. either elective postoperatively or non-elective (Figure 1.1). The score has been shown to not only provide a measure of illness severity, but also correlates with hospital mortality (Knaus et al. 1985).

The APACHE II Severity of Disease Classification System

Physiological variable	High abnormal Range					Low abnormal Range			
	+4	+3	+2	+1	0	+1	+2	+3	+4
Temperature (rectal in ^{0}C)	≥ 41	39-41.9		38.5-38.9	36-38.4	34-35.9	32-33.9	30-31.9	≤29.9
Mean arterial pressure (mmHg)	≥160	130 - 159	110 - 129		70 - 109		50 - 69		≤49
Respiratory rate (ventilated or non-ventilated)	≥50	35-49		25-34	12-24	10-11	6-9		≤5
Oxygenation: A-aDO_2	≥500	350-499	200-349		<200				
PaO_2 in mmHG if FIO2<0.5					>70	61-70		55-60	<55
Arterial pH	≥7.7	7.6-7.69		7.5-7.59	7.33-7.49		7.25-7.32	7.15-7.24	<7.15
Serum Sodium (mEq/l)	≥180	160-179	155-159	150-154	130-149		120-129	111-119	≤110
Serum Potassium (mEq/l)	≥7	6-6.9		5.5-5.9	3.5-5.4	3-3.4	2.5-2.9		<2.5
Serum Creatinine (mg/dl) **Double point for acute renal failure**	≥3.5	2-3.4	1.5-1.9		0.6-1.4		≤0.6		
Haematocrit (%)	≥60		50-59.9	46-49.9	30-45.9		20-29.9		<20
WCC (x10^3cells/mm^3)	≥40		20-39.9	15-19.9	3-14.9		1-2.9		<1
Glasgow Coma Score 15-actual score									
Age (years)	45-54 = **2 points**; 55-64 = **3 points**; 65-74 = **5 points**; ≥75 = **6 points**								
Chronic Health	If the patients has a history of severe organ insufficiency or is immuno-compromised: **2 points** for elective post-operative; **5 points** for non-elective or emergency post-operative								

Figure 1.1. APACHE II scoring sheet from Knaus et al. (1985).

The systemic inflammatory response syndrome (SIRS) represents a multifaceted reaction involving inflammatory and anti-inflammatory processes, humoral and cellular reactions and circulatory abnormalities (Hotchkiss and Karl. 2003; Charalampos and Vincent. 2010). The diagnosis and evaluation of severity is made difficult by the non-specific nature of the signs and symptoms of SIRS. However, early diagnosis and stratification of severity is important to enable timely and specific treatment (Charalampos and Vincent. 2010). Biomarkers that reflect the severity and help with the diagnosis of SIRS could be of significant benefit. Other potential uses of biomarkers include prognostication of outcome and the response to therapy including recovery. C-reactive protein (CRP) is such a biomarker widely used in critical care which has been shown to reflect the inflammatory response (Povoa et al. 2005; Schmit and Vincent. 2008) which is not specific to infection alone but merely indicates the presents of an acute inflammatory process (Clyne and Olshaker. 1999). Importantly, the C-reactive protein is not part of the APACHE II score, the SOFA score and the SAPS score is however widely used as marker of inflammation in critical care (Charalampos and Vincent. 2010)

1.1.2. Number of admissions, length of stay, overall cost and morbidity/ mortality

Current figures from the Intensive Care National Audit and Research Centre (ICNARC) estimate ~ 110 000 admissions per year to general adult intensive care units in England (2006). The estimated cost for Level 2 care per patient per day is £1345 and for Level 3 care the cost is £ 1716 per patient per day. The average care delivered in general adult ICUs has been estimated as 76% at level 3 care and 24% at Level 2 care. Average length of stay is 4.9 days, which gives an average cost of £7972 per patient per admission. Based on the above figures the annual cost for patients admitted to critical care is approximately £ 876 920 000 per year (Personal correspondence with ICNARC. 2006).

Harrison et al (2004) published a case mix study of general adult critical care units from England, Wales and Northern Ireland. This study reported a median age of 63 years; 59% of these were male. The mean APACHE II score was 16.5. Critical care mortality was 20.3%; mortality at discharge from hospital was 30.8%.

1.1.3. Pathophysiology of critical illness – The role of oxidative stress and the inflammatory response

During aerobic oxygenation O_2 is reduced to H_2O at the end of the mitochondrial respiratory chain. However during mitochondrial respiratory oxygenation single electrons leak causing partial reduction of O_2 to the superoxide anion $O_2^{\cdot}$ (Frei. 1994). Superoxide is a radical with an unpaired electron and, as it lacks an electron, highly reactive. Superoxide is therefore called a reactive oxygen species (ROS). Superoxide occurs normally during aerobic oxygenation (Frei. 1994).

In health, there is a balance between the formation of ROS and their effective removal by protective antioxidants before they damage critical biological molecules. Under normal circumstances this balance is maintained between reactive oxygen species (potentially causing cell and tissue damage) and protective antioxidant mechanisms, removing these reactive oxygen species (Gutteridge and Mitchell. 1999).

Apart from appearing as products of aerobic oxygenation, ROS have several important functions in health and disease. Regulation of vascular tone, sensing of oxygen tension and regulation of functions that are controlled by oxygen concentration have been found to be physiological functions of ROS (Dröge. 2002). Further, ROS enhance signal transduction and modulates physiological responses in muscle cells after contractile activity. For example, the activity of antioxidant enzymes was increased by the modulation of the gene expression (Jackson. 2009).

ROS play an integral part in host defence. ROS has been linked to the activation of polymorphonuclear leukocyte and other reticulo-endothelial cells after exposure to infectious matter or after cell/ tissue injury (Brown et al.

2006; Gutteridge and Mitchell. 1999; Marshall. 2005). For example ROS enhance signal transduction from the antigen receptor of lymphocytes (Dröge. 2002).

Neutrophils and monocytes as well as producing ROS during aerobic oxygenation, contain soluble oxidase enzymes with which molecular oxygen can be transformed into ROS (for example hydrogen peroxide and peroxynitrite). These ROS are released into phagosomes or into the extracellular environment and have the ability to damage biological macromolecules for example cellular membranes and lipoproteins, therefore "digesting" pathological microorganism by direct damage of ROS (Frei .1994; Marshall. 2005). The increased concentration of ROS is thought to augment the degradation of pathogens, might however by the same mechanism cause tissue damage to any other tissue in close proximity (Holman and Seba. 1988).

Oxidative stress is represented by a mismatch of ROS and protective antioxidants and scavenger enzymes resulting in a lack of oxidative protection. It has been widely accepted that critical illness, among other factors, is associated with significant oxidative stress. This imbalance can results in tissue damage, exacerbate organ injury and thus overall clinical outcome by the unopposed activity of ROS (Crimi et al. 2006). Oxidative stress in turn has the propensity to worsen organ injury and therefore worsen overall clinical outcome by direct oxidative damage to cell membranes, lipoproteins and other macromolecules. Evidence suggests a correlation between illness severity and oxidative stress of critically ill patients, i.e. the greater the illness severity (expressed by APACHE score) the greater the oxidative stress (Alonso de Vega et al. 2000).

In critical illness the inflammatory response is a crucial mechanism of the host defence to guarantee integrity and survival of the host organism. This inflammatory response is normally tightly controlled to ensure initiation, maintenance and resolution of the inflammatory process to limit unwanted tissue damage (Figure 1.2; Texereau et al. 2007).

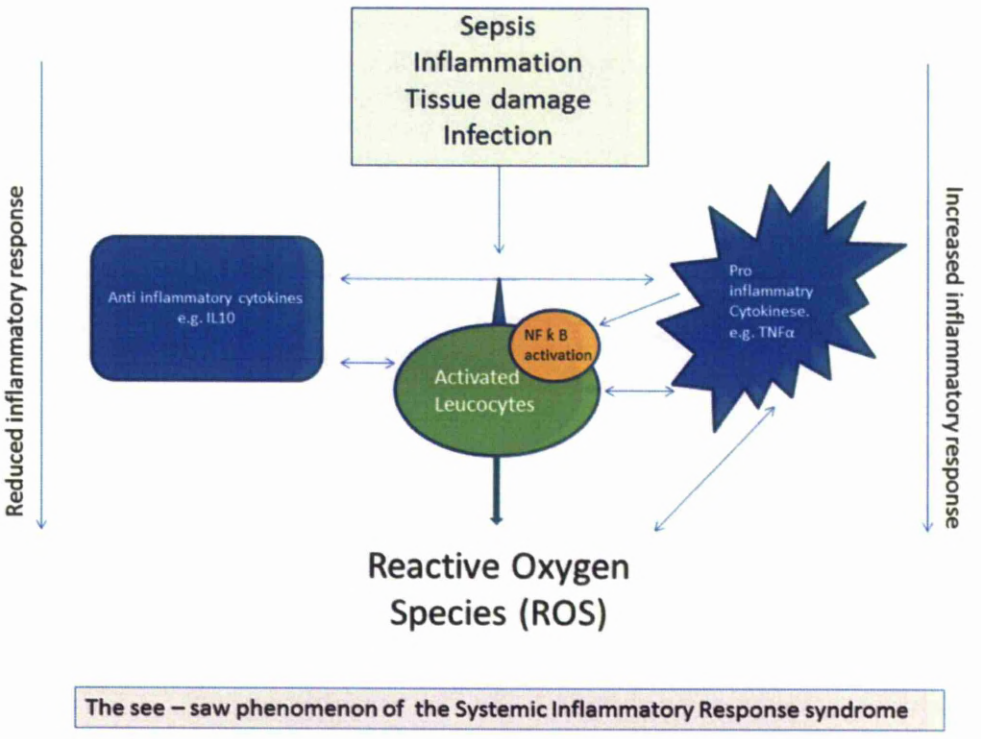


Figure 1.2 Schematic illustration of the Systemic Inflammatory Response Syndrome illustrating the intricate balance between pro and anti-inflammation adapted from Gutteridge and Mitchell, 1999.

The activation of immune competent cells is generated by translocation of the transcription factor of NF ƙ B into the nucleus, as NF ƙ B regulates the expression of genes important for the inflammatory response (Zingarelli. 2005). Cytokines are important proteins to regulate this inflammatory process (Texereau et al. 2007). Activation of polymorphonuclear leukocyte and other reticule-endothelial cells causes early secretion of pro-inflammatory cytokine such as Tumour Necrosis Factor-α (TNF-α), Interleukin 1beta (IL 1beta) and Interleukin 6 (IL 6) which in turn stimulate various cell types and so coordinate the innate and acquired host defence response (Texereau et al. 2007). NF ƙ B can be further stimulated by cytokines such as TNF-α, which plays a central role in this process and provokes a broad range of immune and inflammatory reactions including fever, shock and tissue damage. TNF-α further generates nitric oxide (NO), ROS and cytokine release, since cytokines have the ability to further stimulate the production of additional cytokines.

Following on from above, TNF-α has been suggested to activate and NF ƙ B in turn stimulates the secretion of TNF-α (Figure 1.3; Kaech and Calandra. 2007).

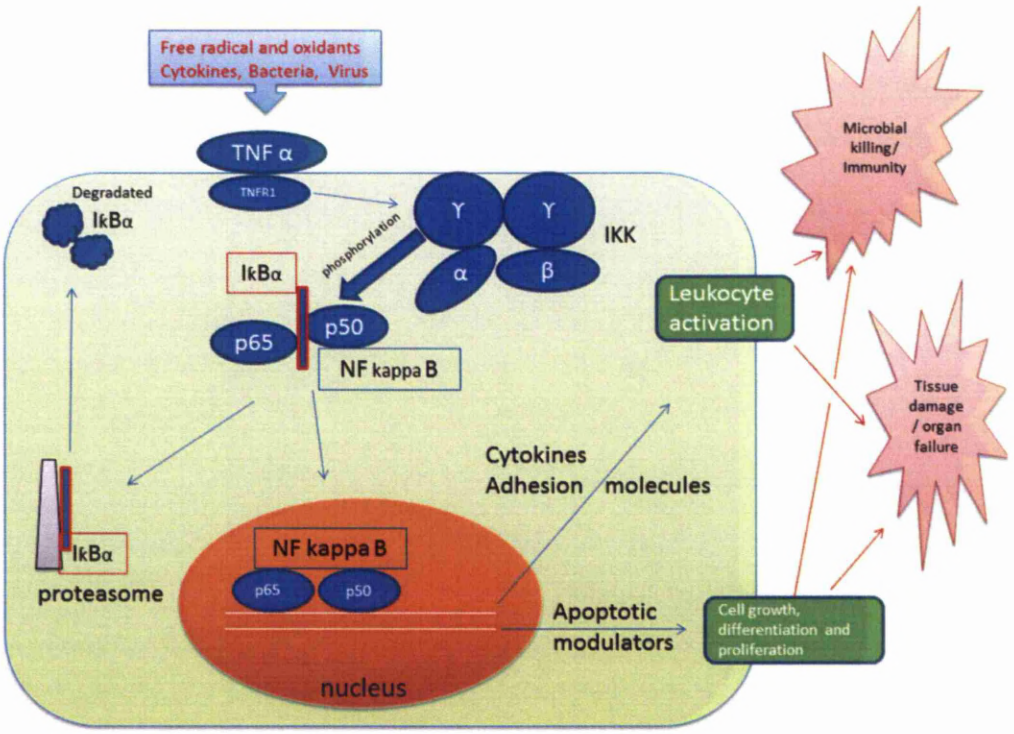


Figure 1.3 Schematic illustration of NF ƙ B activation. A variety of microbial products and inflammatory stimuli activate the inhibitor ƙ B protein kinase (*IKK*) complex, which phosphorylates Iκ Bα associated with NF ƙ B p50/p65 heterodimer. After degradation of Iκ Bα, the NF ƙ B p50/p65 heterodimer is free to translocate into the nucleus to initiate transcription of genes of inflammatory and immune mediators adapted from Zingarelli, 2005.

The basic aim of the inflammatory response is to neutralise and eliminate the affecting agent and to repair the initial injury. Failure to achieve this will lead to chronic inflammation (Bellingan. 2007). An overwhelming inflammatory response can cause tissue damage, as outlined above (Holman and Seba. 1988). It is therefore extremely important to tightly control such an inflammatory response. This becomes even more crucial when the initial insult has been resolved and resolution of inflammation needs to be rapidly

achieved. This response is mediated by the secretion of anti-inflammatory cytokines such as Interleukin 10 (IL 10). A successful and adequate response to any given insult does therefore depend on an appropriate and balanced inflammatory response (Remick. 2007). This is evident by the fact that anti-TNF-α therapy did not produce a beneficial effect in severe critical illness (Abraham et al, 1998; Panacek et al, 2004). Similarly it is widely accepted that non-selective anti-inflammatory treatment with high dose steroid therapy carries no mortality advantage in severe sepsis (Keh and Sprung. 2004). Similarly a blunted TNF-α response conferred a mortality disadvantage (Heagy et al. 2003) possibly worsened by an elevated IL 10 response (Rigato and Salomao. 2003).

1.1.4 Critical illness and nutrition

The case-mix of patients admitted to critical care ranges from those admitted electively after major elective surgery to those admitted as emergencies after some surgical catastrophe for example burst abdominal aneurysm, gastrointestinal perforation, pancreatitis, major trauma, sepsis or respiratory failure (Harrison et al. 2004)

The variation in age range and previous health status may be extreme, and critical care units are now admitting increasingly more elderly, frail or malnourished patients whose nutritional reserve may be severely compromised (Griffiths and Bongers. 2005; Harrison et al. 2004). Critical care patients suffering from under nutrition with a limited nutritional reserve have a poorer outcome (Giner et al. 1996). Furthermore, having a low body mass index has been shown to be an independent predictor of excess mortality in multiple organ failure (Uehara et al, 1999). Critical care has developed significantly since the 1960s with the advent of more sophisticated techniques to support organ failure. Gastrointestinal failure requiring parenteral nutrition is a common problem of up to 10% of patients with critical illness (Reintam et al. 2006). Parenteral nutrition has been established as a treatment modality since the 1960s (Vinnars and Wilmore. 2003). Therefore nutritional support in general and specific nutritional compounds such as the amino acid glutamine

has gained increasing interest in critical care medicine with the hope of preventing or attenuating the effects of malnutrition and nutrient depletion.

1.2. Interactions of glutamine, skeletal muscle and critical illness

1.2.1. Glutamine

Glutamine is the most abundant free amino acid in the body. Glutamine a five-carbon amino acid containing two carboxyl groups one of which is bound to nitrogen, forming an amide group (Raymond D'Souza and Tuck. 2004; Young and Ajami. 2001; Figure 1.4).

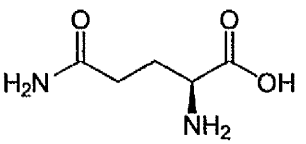


Figure 1.4 Schematic illustration of L-glutamine. (Koolman, 1994).

In the extracellular fluid, glutamine constitutes about 25% of the free amino-acid pool and in skeletal muscle glutamine constitutes more than 60% of the free amino-acid pool (Raymond D'Souza and Tuck. 2004; Young and Ajami. 2001).

Glutamine has a wide range of functions (Koolman and Roehm. 1994; Biolo et al. 2005; Figure 1.5):

- substrate of protein synthesis
- controls acid-base balance via renal ammonia genesis.
- substrate for hepatic urea genesis
- substrate for gluconeogenesis
- fuel for enterocytes
- precursor for nucleic acid
- substrate for citrulline and arginine synthesis
- L-Arginine-NO-metabolism
- glutamine functions as ammonia scavenger

- nitrogen donor
- nitrogen transport
- glutamate transport
- substrate for glutathione production.

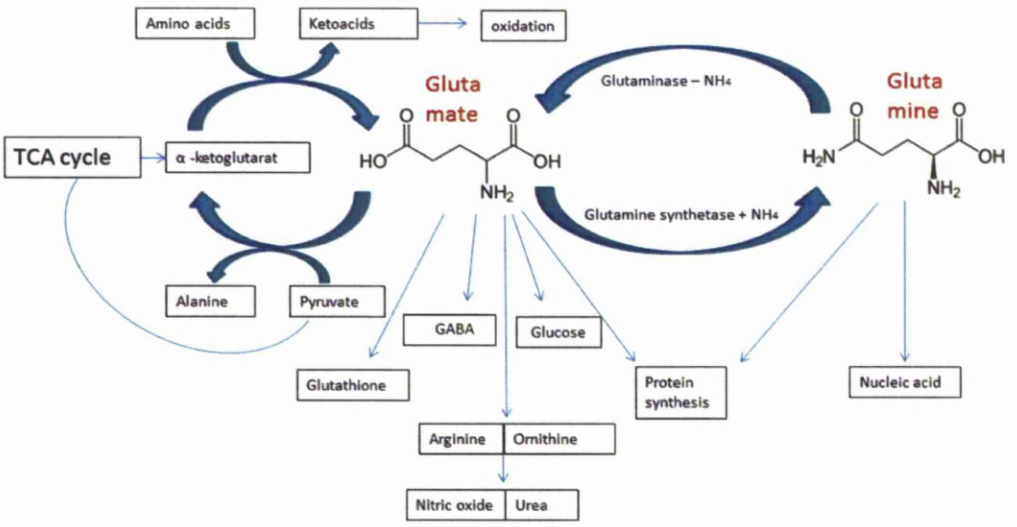


Figure 1.5 Illustration of glutamine metabolism and function. Adapted from Biolo et al. 2005 and Koolman and Roehm. 1994. GABA: gamma-amminobutyric acid, TCA cycle: Tricarboxylic acid cycle.

1.2.2 Skeletal muscle

Skeletal muscle has several main functions. It converts chemical energy into mechanical - and heat energy, which in turn can be used for movement, postural support and heat to maintain body temperature. Due to its specialised structure, muscle is able to contract, which facilitates movement and postural support (Silbernagel and Despopoulus. 1988).

Skeletal muscle contains bundles of individual muscle cells (= fibres) of 10 – 100μm diameter and up to 20 cm length. Skeletal muscle fibres are surrounded by a plasma membrane called sarcolemma and contain multiple nuclei. The sarcolemma further encloses myofibrils. These myofibrils contain

myosin and actin filaments. The arrangement of these contracting protein filaments is the cause of the striate appearance of skeletal muscle. On the one side the actin filaments are fastened to connective tissue, called the Z disk; on the other side, six actin filaments surround one myosin filament. The area where the myosin and actin filaments overlap appears darker and is called A band (Silbernagel and Despopoulus. 1988).

During a contraction the actin and myosin filaments glide along each other and therefore reduce the distance between the Z disks. The muscle contracts and shortens. This process is initiated by an action potential causing a rapid influx of Ca^{++} which binds to Troponin. The Ca^{++}/ Troponin binding frees the myosin/ actin binding site from tropomyosin. Actin and myosin bind under the influence of ATPase which reduces ATP attached to the myosin filament. This ATP loss causes a structural change to the myosin filament which results in muscle contraction. ATP is required to release the actin/ myosin binding (Silbernagel and Despopoulus. 1988).

1.2.3 Effects of critical illness on skeletal muscle

In mammalian cells proteins are constantly exchanged. The average rate of protein replacement varies greatly between tissues. The amount of intracellular protein replacement each day is sizeable. In a normal 70-kg adult, about 280 g of protein is synthesized and degraded each day; the majority of these proteins are intracellular proteins (Mitch and Goldberg. 1996; Figure 1.6).

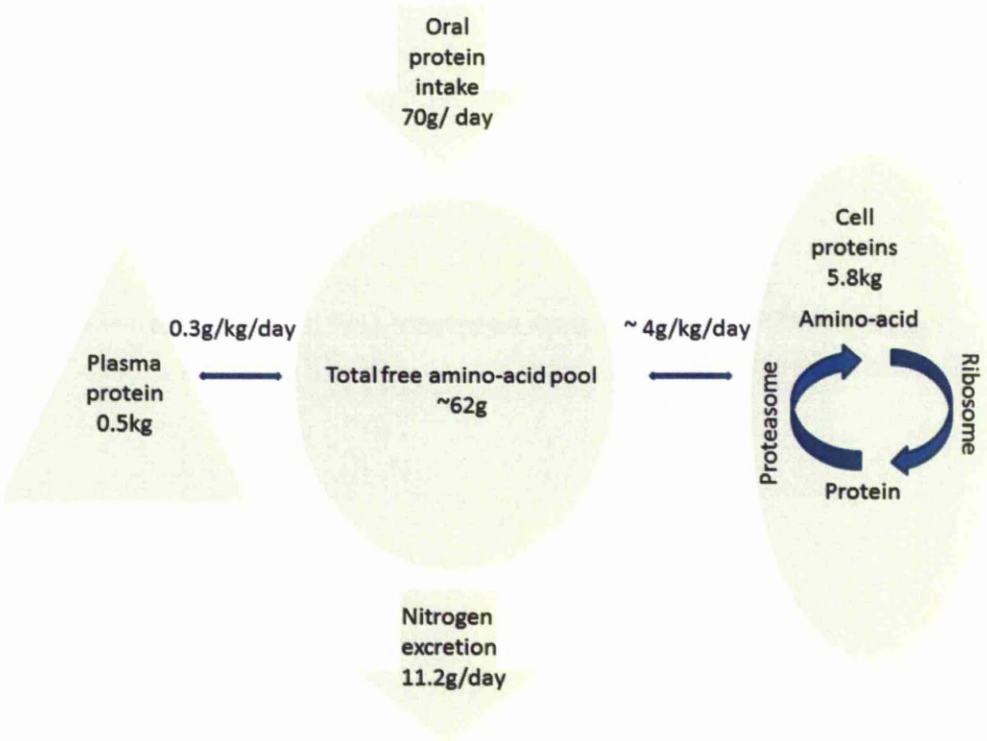


Figure 1.6 Turnover of cellular and plasma proteins in a normal 70-kg man. (adapted from Mitch and Goldberg. 1996).

In catabolic states of sepsis or SIRS the overall breakdown of cell proteins increases to provide the essential amino acids required for protein synthesis and energy metabolism. Importantly during sepsis and SIRS there is a preferential loss of protein from skeletal muscle, but visceral organs lose little or no protein and the brain appears to be unaffected (Mitch and Goldberg. 1996). The muscle wasting is further caused by disuse and a disease process called "critical illness polyneuromyopathy". As suggested by the name, the underlying pathology can either cause an axonal poly neuropathy or a myopathy of some sort. It has been suggested that between 40 – 50% of critically ill patients suffer from critical illness polyneuro-myopathy. It has recently been suggested that critical illness myopathy and critical illness neuropathy coexist frequently (Schweickert and Hall. 2007; Khan et al. 2006) and are possibly different manifestations of the same pathology (Schweickert and Hall. 2007). The muscle wasting that occurs in catabolic states like sepsis or SIRS or following polyneuro-myopathy results largely from the accelerated breakdown of muscle protein, causing muscle wasting during critical illness.

Amino acids are released from muscle tissue (Gamrin et al. 1996), of which alanine and glutamine compose 50% to 70% of the amino acid nitrogen released (Brooks et al. 1986). All of the above mentioned mechanisms – catabolic state, disuse, neuropathy and myopathy - cause significant changes to striate muscle during critical illness and indeed during critical illness mean fibre cross-sectional area is reduced by 3-4% per day (Helliwell et al, 1998). Further, protein content of muscle is reduced by 12% (Gamrin et al. 1997) overall body protein content is reduces by 15% compared with the normal.

1.2.4 The relationship between critical illness, glutamine and skeletal muscle

Glutamine is a non-essential amino acid and as such was not part of the available total parenteral nutrition regimes 'in' the 1960s. This was further influenced by the technical difficulties in the preparation and storage of glutamine containing solutions. L-glutamine has a very poor solubility, is not heat stable and therefore has to be stored at $\leq 4^{\circ}C$. Since glutamine is readily synthesised in most tissues and therefore classified as a dietary non-essential amino acid, it was considered appropriate to omit from total parenteral nutrition (TPN) regimes.

Although this is certainly true in the healthy human being, it has been suggested otherwise during situations of extreme stress, particularly stresses of prolonged duration. The mechanism for the reduction of muscle glutamine represents a demand for increased rates of glutamine utilisation at the whole-body-level and a relative impairment of *de novo* synthesis in skeletal muscle leading to a failure of systemic delivery to other organs and a conditional deficiency (Biolo et al. 2005). The lung and the brain also produce glutamine but skeletal muscle, by virtue of size and ability to synthesise glutamine *de novo* seems to be the most important source of glutamine for the blood stream (Figure 1.7).

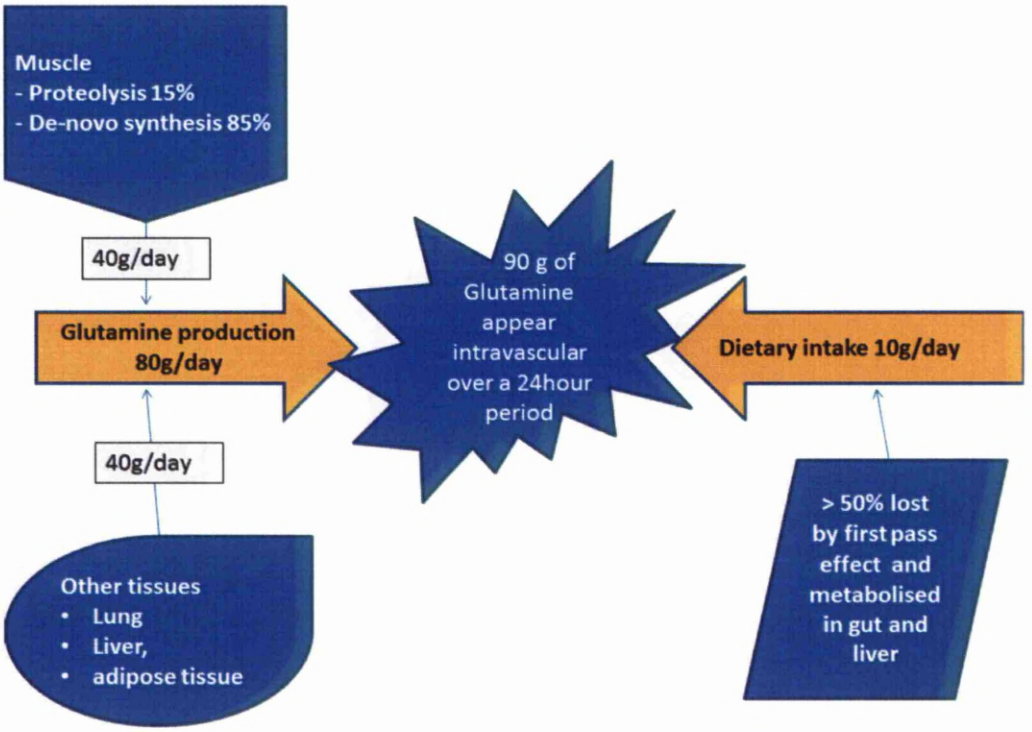


Figure 1.7 Schematic illustration of the source of glutamine illustrating muscle as the major glutamine source for intravascular glutamine. (Adapted from Biolo et al, 2005).

The initial response to septic stress is to export glutamine to the splanchnic bed and immune system from the free amino acid pool in muscle (Biolo et al. 2005). This leads to protein breakdown and *de novo* synthesis of glutamine where *de novo* synthesis of glutamine may represent as much as 85% of the released glutamine (Kuhn et al. 1999). TNF-α (Chakrabarti. 1998) and endotoxin (Elgadi et al.1998) have been shown to increase glutamine synthetase mRNA in both lung and muscle, but in animals with loss of muscle mass due to starvation the increases in glutamine synthetase protein levels are seen predominantly in lung tissue signifying its greater importance when muscle mass is diminished.

Enteral dietary glutamine is extracted and utilised by the gut and liver (Haisch et al. 2000). Elsewhere in the body, on the arterial interface, glutamine rather than glutamate is the major transported substrate across cell membranes. This may be the key to the importance of the systemic plasma levels and

(parenteral) delivery of glutamine for many tissues. Not only does the human body synthesise glutamine in many tissues it also holds it free in solution in skeletal muscle at a gradient of 32:1 muscle: plasma levels by active transport mechanisms (Biolo et al, 2005).

Vinnars et al (1975) were first to observe that following surgery, trauma or sepsis the free glutamine pool in muscle was reduced. Despite the rapid fall in the intramuscular concentration of free glutamine, transport out of muscle was maintained and clearance from the plasma by other tissues increased, indicating activated transport mechanisms in muscle (Mittendorfer et al, 1999). Detailed stable isotope studies of glutamine metabolism in critically ill patients support the extensive and robust data of large animal studies that show net flux of glutamine from skeletal muscle to vital organs (Biolo et al. 2005; Newsholme. 2001; Kuhn et al. 1999)

Jackson *et al* (1999) demonstrated that in newly admitted ICU patients, a similar production rate occurred but an increased metabolic clearance rate of glutamine from plasma also occurred, consistent with increased utilisation by other tissues and only modest correction of low plasma levels with 28 g/day of glutamine infusions. Later on during an intensive care stay, the efflux of glutamine cannot be maintained and plasma delivery declines (Biolo et al, 2000). In children with burns the plasma glutamine levels were reduced despite an increased metabolic turnover of glutamine and a near normal net efflux from the leg suggesting a relative inadequate production in critical illness (Gore and Jahoor. 2000). A decrease in muscle glutamine production was confirmed by Biolo et al (Biolo et al. 2000) in a study of 20 severely burned patients two weeks into their critical illness. The glutamine deficiency appears to be maintained even when moderate amounts of glutamine are supplemented parenterally (Palmer et al. 1996). A further study investigated the dose response of parenteral glutamine supplementation. Plasma glutamine concentration of patients responded by normalisation of plasma glutamine levels in a dose-dependent way, while free muscle glutamine concentration, as well as muscle protein synthesis and muscle protein content did not change significantly (Tjäder et al. 2004). There is therefore strong evidence to contend that glutamine should be regarded as a 'conditionally essential' amino acid in critical illness. More importantly the question remains as to whether a

low plasma and/or tissue glutamine concentration has an effect on morbidity or mortality. This question was addressed by an observational study from the Netherlands (Oudemans-van Straaten et al. 2001). In this study, plasma glutamine was measured in 80 critically ill patients. A low glutamine concentration was associated with higher mortality and a trend towards higher severity scores. The low plasma glutamine group was considerably older, possibly consistent with a reduced muscle bulk. The same findings were reproduced, i.e. low glutamine concentration was associated with higher mortality in a further study from Rodas et al (2012).

Work from other researchers (Wischmeyer. 2006b) has suggested that glutamine may work as a "pharmacological" agent as it conveys a morbidity and mortality advantage in a dose dependant pattern i.e. the higher the glutamine concentration given the greater the morbidity and mortality advantage (Wischmeyer. 2006b). To support this theory the researchers treated male Sprague-Drawley rats with varying doses of intravenous glutamine, followed by an endotoxin infusion to induce sepsis. A morbidity and mortality advantage the greater the glutamine dose administered was observed (Wischmeyer et al. 2001). Further, a mortality advantage was equally observed in several clinical studies (Griffiths et al. 1997; Powell-Tuck et al. 1999; Wischmeyer 2001). On the basis of the above studies a systematic review therefore suggested a beneficial glutamine effect on mortality the greater the dose administered (Novak et al. 2002) which was further supported by clinical practise guidelines (Heyland et al. 2009). However, this does not preclude these effects alongside that of correcting a deficiency. These two approaches - pharmacological effect or correcting a conditional deficiency - are obviously not mutually exclusive (Kent and Bongers. 2011).

1.2.5 Clinical outcome

The important question is whether exogenous glutamine provision affects outcome. However to understand the available data requires an appreciation of the timescales and consequences that could reasonably be expected. It is apparent when caring for patients on intensive care that some patients die

early from the failure of a single organ that was involved in the primary pathology; and this usually involves just the brain, the heart or the lung. While patients die later from multiple organ failure which is "associated" with the development of secondary infections and is often a combined system failure (e.g. lung, liver and kidney) and is more a feature of non-recovery from initial or maintained insults. For instance although an acute inflammatory response is an early feature of most ICU presentations it is now appreciated that an optimised immune system, still capable of mounting normal inflammatory signalling, is a feature of survival. Perhaps the greatest challenge with any nutritional replacement therapy is to appreciate the likely time scales involved to show a clinically meaningful response. A benefit will likely be greatest in those who are most deficient for a prolonged period, sufficient to affect many systems e.g. the most severely ill with gut failure dependent solely on a glutamine deficient parenteral nutrition. Glutamine also needs to be given in sufficient dose and for sufficiently long periods. E.g. low dose continuous enteral delivery may all be consumed in first pass metabolism and not continuing glutamine after the initial treatment period in those not recovering within intensive care. Giving too little for too short a treatment period may well have little impact on survival, furthermore choosing an early endpoint e.g. 28 days may not disclose a real effect. Evidence suggests it is therapies that conserve or restore optimal circulation (Rivers et al. 2001) and metabolism (Van Den Berghe et al. 2001) that have shown real outcome benefit. Providing the optimal nutrition environment by preventing deficiency arising (Griffiths. 2001) is central to improved survival from Multi Organ Failure (MOF) (Griffiths. 2004)

1.2.6 Glutamine supplementation

1.2.6.1 Enteral supplementation

The evidence for enteral glutamine substitution, i.e. administration via the normal gastrointestinal route either by eating or a feeding tube, is currently not convincing. A large study from Western Australia randomised 363 relatively well nourished critically ill patients to an enteral supplement of

about 19g of glutamine per day (Hall et al. 2003). Neither mortality (glutamine 15% (27/179) versus control 16% (30/184)) nor severe sepsis incidence (glutamine 21% (38/179) versus control 23% (43/184)) were affected. Typical of critical care, about 8% of patients required parenteral nutrition and did not receive the feed. The lack of any effect on mortality seems to be an observation consistent with previous lower dose enteral glutamine studies and may reflect the limited systemic availability of glutamine through the enteral route for it to be sufficient in the sicker patients to significantly influence survival (Haisch et al. 2000; Melis et al. 2005). This is probably not surprising as rapidly dividing cells (enterocyts) are using glutamine as energy source, so readily utilise enterally supplied glutamine. This would suggest that glutamine given enterally will not or to a lesser amount appear in the systemic circulation, which in turn may be the reason for the limited effect of glutamine on clinical outcome. This could be convincingly shown in a study comparing enteral with parenteral glutamine administration (Melis et al. 2005). In this study, 20 g of alanine-glutamine dipeptide infusion was given to patients over 4 hours. Plasma glutamine concentration rose significantly with enteral and parenteral administration, but significantly more so when administration was parenteral. These findings would suggest the need for relatively higher glutamine doses when given enterally, to achieve the same systemic concentrations. Indeed researchers have shown that 14 day enteral supplementation in severe burns patients with 0.5g/kg bodyweight of glutamine granules compared with placebo showed significantly increased plasma glutamine concentrations, improved wound healing and shortened hospital stay (Peng et al. 2004; Peng et al. 2006).

It has been postulated that the severity of illness correlates with the need for glutamine substitution (Novak et al. 2002, Heyland et al. 2009). This could be a further reason why enteral glutamine supplementation does not appear to carry a significant benefit compared to parenteral supplementation as severity of illness appears to correlate with gastrointestinal failure (Reintam et al. 2006) i.e. patients with intact gastrointestinal function are less ill (Kent and Bongers 2011).

1.2.6.2 Parenteral supplementation

Various trials have investigated the role of parenteral glutamine administration in critical illness, i.e. the intravenous administration of glutamine bypassing the gastrointestinal tract (Griffths et al. 1997, Powell-Tuck et al. 1999, Goeters et al. 2002, Déchelotte et al. 2006). One of the earliest trials examining glutamine administration in critical illness in a randomised controlled fashion was published by Griffiths et al (1997). These authors investigated a group of 84 severely ill critically care patients who were unable to receive enteral feeds and where major sepsis was the predominant feature. In a double blind manner, patients were randomised to glutamine containing total parenteral nutrition (TPN) or iso-caloric and iso-nitrogenous TPN without glutamine. On an intention to treat basis a significant reduced mortality benefit after 6 months in the glutamine group was observed, an outcome chosen to better reflect the known timescales of recovery of such patients. Survival in the glutamine group was 24/42 compared with 14/42 in the control group (Griffths et al. 1997).

Using previously unpublished data, the same authors (Griffiths et al. 2002) showed that glutamine recipients have a significantly lower risk of dying from intensive care acquired infections. The difference in survival noted was almost all explained by reduced intensive care mortality from multiple organ failure in those patients remaining in ICU for a longer period and requiring at least five days of parenteral feed ($p=0.05$). In these very sick patients colonisation with Candida was high but fewer glutamine recipients acquired infections after a longer time on feeding and none died, whereas six control patients acquired Candida infections and died from multiple organ failure ($p=0.02$). This is a clinical illustration of how glutamine may restore the impaired T-cell mediated acquired immunity and allow optimal recovery of immune competent cells suffering from glutamine deficiency (Eliasen et al. 2006). There soon followed a further parenteral study (Powell-Tuck et al. 1999). In this study 168 patients with the need for parenteral nutrition were randomised to either glutamine, supplemented TPN or control TPN of iso-caloric and iso-nitrogenous values. This study could not show a significant difference in 6 month mortality or infectious complications. However there was a non-

significant trend towards improved in-hospital mortality in medical and haematological patients in the glutamine-treated group. A major criticism of this study was a lack of patient homogeneity, even more so than usually observed in critical care studies (Carson and Shorr. 2003). Fewer very ill intensive care patients, represented by a lower mean APACHE II score, were recruited. However, it has been postulated that the very ill intensive care patients group are likely to be at the highest risk of glutamine deficiency (Griffiths et al. 2002) and will therefore benefit more from parenteral glutamine administration. Excluding very ill intensive care patients could therefore reduce any observed benefit.

Two further studies used the dipeptide alanyl-glutamine to overcome the solubility and stability issues of glutamine. 114 ICU patients in a French multi-centre randomised controlled trial (RCT), of predominantly complicated surgery or trauma patients showed on intention to treat analysis a significant reduction in complicated outcomes (41.4% v 60.7% $p<0.05$) that was predominately related to reduced infectious rate and pneumonias (Déchelotte et al. 2006). There was however no survival difference in the two groups. The median duration of feed was about 6-7 days; the maximum feed duration however was limited to only 10 days. This may be critical to any effect on outcome since it is possible that a longer period of treatment is required for an effect on survival to become apparent in those who were most deficient. The timescale effect was emphasised by a study involving 144 ICU patients in Germany (Goeters et al, 2002). In this randomised but un-blinded study the investigators decided *a priori* to analyse the data of 95 patients treated for more than five days and 68 patients treated for more than nine days. Plasma glutamines were low as expected and even by five days had not returned to normal since the feed only contained 0.2 g/kg body weight of glutamine compared with 0.35g/kg used in earlier studies. There was a small but non-significant difference in clinical outcome in those patients fed for a shorter period but for those fed for more than nine days the survival measured at six months was significantly improved 22/33 v 13/35 ($p<0.05$).

A systematic review tried to address the important criticism of the paucity of trials and the small number of patients involved in these trials (Novak et al. 2002). The authors examined randomised controlled trials of glutamine

supplementation in surgical and critically ill patients using electronic databases and identified 14 trials. When aggregated, glutamine supplementation was associated with a risk ratio (RR) of 0.78 (95% confidence interval [CI], 0.58–1.04) for mortality. Glutamine supplementation was further associated with a lower rate of infectious complications (0.81; 95%, 0.64–1.00) and a shorter hospital stay (2.6 days; 95%, 4.5 to 0.7). The mortality benefit was even more striking in the trials that used the parenteral route (0.71; 95%, 0.51–0.99) and higher glutamine concentrations (0.73; 95%, 0.53–1.00). The addition of trials published more recently has not altered the overall conclusion and when the 3 level 1 and 5 level 2 studies were aggregated, glutamine supplemented PN was associated with a significant reduction in mortality in critically ill patients (RR 0.67, CI 0.48, 0.92, p = 0.01) using a range of glutamine of 0.2-0.57 g/kg/day (Heyland et al. 2009). However, these findings have been questioned by three further trials that have been published in 2011. Grau et al (2011) demonstrated that glutamine supplemented TPN was associated with a reduced rate of infectious complications. These authors identified 127 patients with APACHE II score >12 requiring TPN for 5-9 days. TPN was supplemented with $0.5g/kg^{-1}/day^{-1}$ alanine glutamine dipeptide (Ala-Gln). This was associated with fewer urinary tract infections compared with the control group (2.3% versus 16.9% urinary catheter days, p=0.03) and fewer episodes of nosocomial pneumonia (8 versus 29 % days of mechanical ventilation, p=0.02). There was no statistical difference in intensive care mortality, hospital mortality and 6 month mortality. The Scandinavian Glutamine Trial investigated 413 ICU patients with an APACHE II score > 10 at admission (Wernerman et al. 2011). The patients were given enteral and/ or parenteral nutrition supplemented with iv glutamine for 12 hours per day. This was associated with a statistically significant increase in survival at 28 days in the group supplemented with iv glutamine, but no improved mortality at six months. The trial was stopped early due to recruitment problems and lack of funding, and was therefore unable to come to a definitive conclusion.

The largest published trial to date of glutamine supplemented TPN is the SIGNET trial which recently investigated 502 adults receiving HDU or ITU care at 10 centres in Scotland (Andrews et al. 2011). This study randomized

patients to TPN containing glutamine, selenium or both in adult patients expected to require high dependency or intensive care for ≥ 48 hours and required ≥ 50% of their nutritional requirements to be met by parenteral nutrition. Patients randomised to glutamine received TPN supplemented with 20.2g glutamine per day. Participants had a median APACHE II score of 20. 59/126 (47%) of the patients received the glutamine supplemented trial TPN for < 5 days (median 5.1 days, max duration 7 days). This study could not show any significant benefit of parenteral glutamine supplementation with regards to infection rate or mortality. The negative results of this trial are however not surprising considering the small dose and short duration glutamine was administered.

1.2.7 Functional effects of glutamine

1.2.7.1 Effect of glutamine on glucose metabolism

It has been suggested, that glutamine, as parenteral infusion, can beneficially influence insulin mediated glucose utilisation, suggesting that sensitivity or responsiveness to insulin was enhanced (Borel et al. 1998) and in healthy adults can beneficially influence post-prandial insulin action, glucose disposal and fat oxidation (Iwashita et al. 2006). These findings reinforced the hypothesis that glutamine is beneficial during clinical situations associated with insulin resistance, which is frequently seen in critical illness. Two randomised controlled trials investigated this hypothesis further. In the large French RCT study discussed earlier (Déchelotte et al. 2006) in addition to demonstrating a substantial reduced infection and pneumonia rate in glutamine supplemented patients the investigators found a significant reduction of hyperglycaemias and a significant reduction in the number of patients requiring insulin. A further study examined insulin resistance in trauma. The investigators randomised 40 multiple trauma patients to receive either 0.4g glutamine per kg bodyweight per day or iso-caloric and iso-nitrogenous control (Bakalar et al. 2006). To assess insulin sensitivity eu-glycaemic clamp was performed on day 4 and day 8. The investigators showed that improved insulin sensitivity in multiple trauma patients was

positively associated with parenteral glutamine supplementation. Grau et al showed improved glycaemic control in patients receiving TPN containing glutamine with a 54% reduction of the amount of insulin required for the same level of plasma glucose (Grau et al. 2011). In the light of these studies it is reasonable to accept that glutamine has a beneficial effect on insulin dependent glucose metabolism. This is clinically relevant when we look at the current evidence on glycaemic control in surgical and medical intensive care patients and its importance in view of morbidity and mortality (Van Den Berghe et al. 2001; Van Den Berghe et al. 2006; Langouche et al. 2007).

1.2.7.2 Effect of glutamine on physiological and pathological mechanisms of infection

Critically ill patients are at increased risk of sepsis which is a major cause of mortality in critical care (Hotchkiss and Karl. 2003). Moreover a significant number of patients are admitted to critical care due to sepsis as primary diagnosis (Hotchkiss and Karl. 2003). It is therefore not surprising that sepsis and its avoidance is a major focus in current critical care research. It has been suggested for some time now that glutamine is a major fuel supply for immune cells. This appears to be the case for all immune competent cells. Moreover, optimal phagocytic and secretory activity of immune cells may be dependent on adequate glutamine supply (Andrews and Griffiths. 2002). Oehler et al (2002) investigated the effect of glutamine depletion on human leucocytes. These authors found that lymphocytes were less able to produce an adequate adaptive heat shock protein response to hyperthermia with a reduced glutamine concentration of 0.125mM, which is 25% of the physiological glutamine level, at the time of the stress response (Oehler et al. 2002). The authors could further show an adaptation and recovery of monocytes with increased glutamine concentrations after a time of glutamine starvation, however no adaptation was observed at continuously low (33%) glutamine concentrations (Eliasen et al. 2006). A further study investigated the role of glutamine and peripheral blood polymorphonuclear cells (PMNC). The investigators showed that increased glutamine levels greater than 4mM

reduced the TNF α release from blood PMNCs after 4 and 24 hours of lipopolysaccharide (LPS) stimulation (Wischmeyer et al. 2003).

Recent evidence suggests that glutamine might play a role in NF ƙ B activation. Cells were exposed to LPS to provoke an inflammatory stimulus in a cell culture model containing varying concentrations of glutamine. The researchers found an inverse correlation between NF ƙ B activation and glutamine concentration in media (Yu-Chen et al. 2012).

Enteral and parenteral glutamine supplementation is associated with reduced infectious morbidity in critically ill patients, as outlined in a recent review (Dhaliwal and Heyland. 2005). Various clinical studies have shown a benefit on infectious complications in patients with glutamine supplementation. However, others have not. Therefore glutamine administration either enterally or parenterally remains controversial.

1.2.7.3 Effect of glutamine on glutathione content of tissue

Reactive oxygen species (ROS) are thought to play a key role in the underlying pathophysiology of multisystem organ failure in critically ill patients. When oxygen availability is limited in the tissues of vital organs due to hypoperfusion, the cells shift from aerobic to anaerobic metabolism, thereby lowering the cellular energy charge. As a result, increased adenosine triphosphate (ATP) hydrolysis, a subsequent increase in adenosine monophosphate (AMP) levels and finally, an accumulation of purine metabolites, are found in ischemic tissues. Furthermore, during activation of the immune response, neutrophils, macrophages, and other competent immune cells may activate a plasma-membrane-associated NADPH oxidase system, capable of oxidising NADPH to NAD+, leading to further generation of superoxide radicals. Spontaneous dismutation of the superoxide radical generates hydrogen peroxide and molecular oxygen at physiologic pH. ROS not only lead to direct damage of cellular components, but also trigger the release of cytokines that further activate the inflammatory cascade (Heyland and Dhaliwal. 2006; Grimble. 1994). Glutathione in the reduced form is an important endogenous scavenger of ROS. Glutamine is an important substrate for glutathione. Therefore research has focused on the relationship between

glutamine and glutathione and further, on the effect of low glutamine concentrations on glutathione concentrations in various cells during critical illness (Wernerman. 2003; Fläring et al. 2003). In critical care patients with septic complications following surgery, muscle free glutamine concentration was 72% lower in the sepsis group compared with a healthy control group (Hammarqvist et al. 1997). In parallel, during the first week in critical care, muscle reduced glutathione concentration and total glutathione concentrations were reduced to 57% and 62% of that of healthy controls respectively. Importantly, in this situation there is a significant correlation between muscle free glutamine and muscle total glutathione concentration (Wernerman et al. 1999). In a further study reduced glutathione levels were shown to be reduced in blood in critical care patients (Fläring et al. 2005). The same research group showed that glutamine supplementation attenuated muscle glutathione depletion (Fläring et al. 2003).

1.3 Heat Shock Proteins – Background

Thus, there is considerable evidence that maintenance of plasma glutamine concentrations is beneficial. The mechanisms by which glutamine is beneficial are known to be:

- modification of the glucose metabolism
- manipulation of immune responses
- substrate for the antioxidant glutathione.

A newly emerging function of glutamine supplementation is via the activation of the adaptive and cytoprotective stress, or heat shock response although the mechanisms by which this might occur are poorly understood.

The expression of stress or heat shock proteins (HSPs) is one of the most highly conserved mechanisms of cellular protection and may be central to protect against the assault from systemic inflammation as seen during severe critical illness. Cells have been found to contain increased concentrations of HSPs after exposure to high but non damaging temperatures. This phenomenon was first discovered in 1962 by Ritossa (Ritossa. 1962) and in 1975, Gerner and Schneider showed that the increase in HSP concentration

carried a transient protection against subsequent stress (Gerner and Schneider, 1975). Others have demonstrated that HSPs are increasingly produced after exposure to a wide range of cellular stresses (Kiang and Tsokos. 1998).
HSPs are categorised primarily according to their molecular weight. All HSPs are believed to act as chaperones, some have specific functions and are located in different compartments within the cell. By definition HSP production is increased after exposure to a wide range of environmental stresses and pathological states. Initially, Heat Shock Factor (HSF) becomes activated and enters the nucleus, binds to the Heat Shock Element (HSE) in the promoter regions of HSP genes. This leads to the activation of HSP gene expression and the synthesis of HSPs (Morimoto et al. 1992).

1.3.1. HSP 70

HSP 70s are the most commonly studied Heat Shock Proteins. Two forms of HSP 70 exist. The highly inducible HSP 70 which is significantly expressed during stress and the constitutively expressed but also inducible HSC 70 (Hinault et al. 2006; Bromberg et al, 2007).
HSP 70 is present in the endoplasmic reticulum, mitochondria and cytoplasm and is implicated in a wide range of functions. As molecular chaperones, HSP 70 moderates protein degradation, elimination and repair. HSP 70 supports the correct folding of proteins by an ATP dependant mechanism and is involved in ATP dependant unfolding. HSP 70s are also implicated in cell signalling pathways (Bromberg et al. 2007; Torok et al. 1997).

1.3.2 HSP 60

HSP 60 is located in the mitochondria where it is believed to be involved in protein folding. HSP 60 is believed to catalyse local unfolding of polypeptides in an energy dependant process. HSP 60 is further believed to prevent to proteins aggregation by passively binding of these aggregation-prone proteins to a state that is more readily foldable (Bukau and Horwich. 1998).

1.3.3 The small HSPs

The small HSPs, HSP 27 and α B crystallin are found in the endoplasmic reticulum, mitochondria and cytoplasm are involved in prevention of cellular proteins from aggregation (Torok et al. 1997). Heat shock protein 25 and α B-crystallin are structurally and functionally related small stress proteins induced by a variety of insults, including heat and ischemia. High concentrations of HSP 25 have been reported in cardiac and skeletal muscle. The highest concentrations of α B-crystallin have been reported in eye lens fibre cells, cardiac muscle, striated muscle and lung. HSP 25 and α B-crystallin work as molecular chaperones and are believed to stabilise the cytoskeleton (Smoyer et al. 2000). HSP10 is a small HSP, primarily located and functioning in the mitochondria. HSP10 shares the same chromosome as its co-chaperonin HSP60 and has been associated with simultaneous up-regulation of both genes during instances of cellular stress.

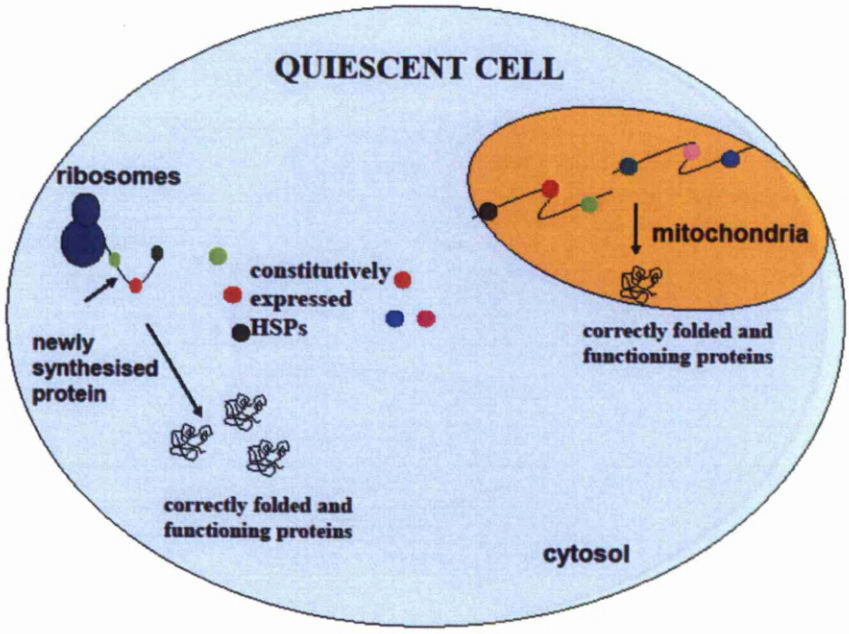
In the mitochondria HSP10 and HSP60 form the chaperonin complex. The chaperonin complex binds and stabilises newly synthesised proteins in the mitochondria and this complex is pivotal in maintaining mitochondrial homeostasis. Studies have indicated HSP10 plays a significant role during cellular stress. Up-regulation of HSP10 is associated with improved outcome in ischaemia injury in cardiac tissue (Dillmann. 1999).

Research from our laboratory has identified a significant role for HSP10 in protection against age-related loss of muscle mass and function, whereby overexpression of HSP10 prevented the age-related loss of force generation (Kayani et al. 2010).

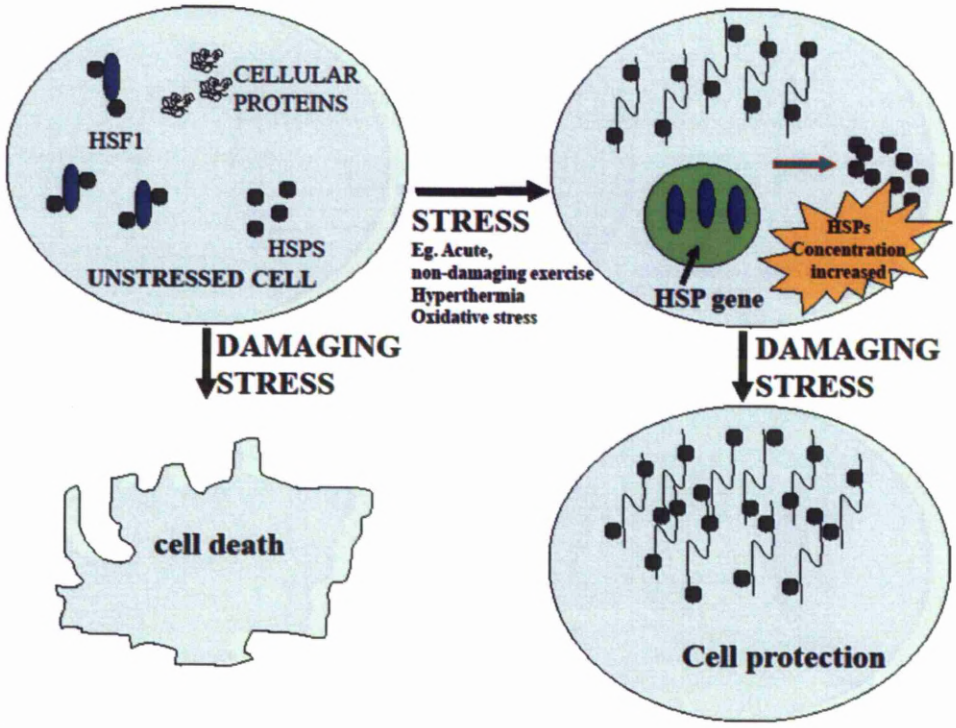
1.3.4. Intracellular HSPs, function in skeletal muscle

All HSPs act to preserve cellular integrity. An adequate HSP response is therefore critical for cell survival. Skeletal muscle normally adapts following stress, such that muscle is protected against subsequent damage (McArdle et al. 2001). This adaptation occurs following a variety of insults. Free radicals are generated, which in turn lead to a rapid adaptive response in the activity of

protective enzymes, such as superoxide dismutase and catalase, and an increase in the cellular content of HSPs. An increase in these protective enzymes and HSPs protects the muscle tissue against subsequent exposure to damage (McArdle et al, 2004). Thus, when induced following cellular stress, HSPs appear to repair denatured proteins or promote their degradation following irreparable injury (Figure 1.8; Hightower. 1991).



a)



b)

Figure 1.8 a) Function of HSPs in the unstressed cell. b) Protective effect of an increased content of HSPs in skeletal muscle. Adapted from McArdle et al, 2002.

The protective effect of increased HSP content in muscle has been demonstrated in a cell culture model comparing heat treated C2C12 myotubes with none heat treated myotubes exposed to cell damaging agents. Cells treated with hyperthermia demonstrated increased HSP content and a significant cytoprotective effect against cell damaging agents (Maglara et al. 2003). A similar protective effect in muscle was demonstrated in an animal model of HSP 70 transgenic mice whereby a transgenic increase in HSP 70 content protected muscle from damaging exercise and improved recovery (McArdle et al, 2004). A further experiment investigated increased HSP content in an animal sepsis model. Animals were randomly assigned to one of four groups: heat treatment with sepsis, no heat treatment with sepsis, heat treatment and sham sepsis and no heat treatment with sham sepsis. Heat treatment resulted in a significant increase in HSP content and in turn a significant mortality benefit (Villar et al. 1994; Chu et al. 1997).

The protective effect of an increased cellular HSP content was very elegantly shown in a severe sepsis model of Sprague-Dawley rats. Sepsis was induced by a two puncture caecal ligation technique. At the time of operation an adenovirus expressing HSP 70 (AdHSP) was administered to the lung via an endotracheal tube. The sepsis-induced lung injury was markedly reduced as was mortality in these animals (Weiss et al. 2002). An inverse correlation was demonstrated between HSP 70 and the cytokines TNF-α, IL 1beta and IL 6 in an animal lung injury model (Vreugdenhil et al. 2003).

1.3.5 Extracellular HSPs

A study in severe trauma patients found a correlation between patient survival and the ability to increase HSP 72 in serum, with a survival benefit in the group with a high HSP 72 content in serum (Pittet et al. 2002). Ziegler et al have shown a possible mortality advantage with an increased HSP 70 serum concentration after intravenous nutritional therapy containing an alanine - glutamine dipeptide (Ziegler et al. 2005). Both studies suggested that the serum HSP content might play a role in survival from critical illness. How this was reflected in tissue levels of HSPs and the mortality advantage was achieved is however not clear. Contrary to intracellular HSPs, extracellular

HSPs not only act as immunosuppressive molecules but also as potent immunostimulator molecules depending on the circumstances by which they interact with cells (Pockley et al. 2008). For example exposure of human monocytes to exogenous HSP 70 resulted in the up-regulation of pro-inflammatory cytokines such as TNF-α, IL 1beta and IL 6 (Asea et al. 2000) and indeed there appears to be a correlation between HSP 70 concentration and severity of inflammation reflected by a correlation between HSP 70 and CRP, HSP 70 and TNF-α and HSP 70 and monocyte count (Njemini et al. 2004). It is further accepted that HSP 70 can activate macrophages, dendritic cells (DC), natural killer (NK) cells, and hepatocytes (Pockley et al. 2008).
Two sources of extracellular HSP have been postulated. HSPs can be released from healthy cells (Hightower and Guidon. 1989) and indeed an active mechanism has been reported (Hunter-Lavin et al. 2004). Others suggested that cell lyses is the main source of extracellular HSP (Basu et al. 2000).

1.3.6 HSP and glutamine

Glutamine appears to regulate protein turnover in cell cultures of myotubes, increasing the half- life of long-lived proteins. This may be related to a glutamine induced increase in HSP 70 (Zhou and Thompson. 1997). Further, glutamine appears to be a potent enhancer of the stress response via HSP expression (Nissim et al. 1993) or increased HSP content in cells (Musch et al. 1998; Wischmeyer et al. 1997). Thus, researchers have demonstrated increased HSP 70 content in tissue of the lung, liver and kidney in a porcine model after recurrent endotoxemia (Klosterhalfen et al. 1997) However, following sepsis, the HSP 70 content of lung tissue was impaired (Wischmeyer and Singleton. 2002), (Weiss et al. 2000) or only marginally increased (Singleton and Wischmeyer. 2006).
The observed attenuated stress response was reversed by glutamine infusion facilitating a significantly greater HSP content of lung tissue compared to placebo (Singleton et al. 2005a). The increased HSP concentration was associated with striking preservation of various organs including lung tissue (Wischmeyer et al. 1997; Wischmeyer et al. 2001). Others have shown that glutamine infusions, over a range of doses (0.15-0.75 g/kg), are able to

enhance HSPs content of multiple organs of the rat (Wischmeyer et al. 2001). This induction occurs as early as 1 hour post-administration and persists for up to 72 hours post-administration. The authors further demonstrated that glutamine infusion prior to a septic insult was associated with protection against endotoxin-induced septic shock in the rat and could markedly decrease end-organ injury and overall mortality. Further experiments illustrated that glutamine given post septic insult enhanced HSP 70 and 25 content, protected against acute lung injury and reduced end-organ injury and overall mortality (Singleton et al. 2005b). The survival benefit from glutamine was abrogated if a HSP inhibitor was administered (Singleton et al. 2005a).

It has been suggested that glutamine deficiency renders the cells incapable of an adequate HSP response (Oehler et al. 2002). Consequently it has been suggested (Wischmeyer 2006a; Wischmeyer 2006b) that glutamine administration influences the HSP response in humans and indeed the observed survival benefit of glutamine added to parenteral nutrition to meet a developing deficiency in the critically ill might reflect this. Moreover it has been demonstrated that glutamine substitution added to parenteral nutrition correlates with increased HSP 70 response in the critically ill (Ziegler et al. 2005).

Half of severely ill intensive care patients are over 65 years with upwards of 25% over 75 years of age. The ability of cells to induce HSPs following stress is reduced in aged humans and animals. Tissues from aged animals and blood cells from elderly humans both show a reduced production of stress proteins following thermal stress (Rao et al. 1999). It has recently been confirmed that an attenuated response occurs in skeletal muscle of aged rodents following a physiological stress. The HSP70 content of resting skeletal muscle was reduced in muscles from aged rodents and the production in response to a period of contractile activity was severely blunted in comparison with young animals (Vasilaki et al. 2002; Vasilaki et al. 2003). This lack of adaptation in HSP content in the aged animals may be related to a more general failure of adaptation to stress. Moreover, as mentioned above it has been observed that a low glutamine concentration might be associated with older age (Oudemans-van Straaten et al. 2001). Further, HSP response in trauma might be blunted in older age (Pittet et al. 2002). Evidence to date is still lacking whether

glutamine supplementation in this high risk group can improve plasma glutamine concentration, HSP responses and ultimately outcome.

1.4 Summary

Over the last 20 years, increasing evidence is emerging to support the use of glutamine supplementation in critical illness. Animal work and clinical trials have found a mortality and morbidity advantage of glutamine supplementation in critical illness. The advantage appears to be greater with increasing glutamine; further, the advantage is greater when given parenterally. Various modes of action have been postulated. Glutamine seems to have an effect on the immune system, the antioxidant status, glucose metabolism and heat shock protein response. It has been shown that increased intracellular and extracellular HSP concentrations convey a protective effect during damaging stress. This could be shown in cell culture models as well as animal models of sepsis and clinical studies of critical illness. Research has therefore concentrated in finding safe mechanisms to augment the HSP response particularly in skeletal muscle to improve clinical outcome.

1.5 Hypothesis

The overall hypothesis of the work is that:

1. Lower than adequate levels of extracellular glutamine will result in an altered ability of muscle cells to produce HSPs at rest and in response to physiological stress.
2. Higher than normal levels of extracellular glutamine will result in no additional ability of muscle cells to produce HSPs at rest and following a physiological stress compared with adequate levels of extracellular glutamine.
3. Further, that glutamine administration will have a direct effect on the HSP content of muscle of critically ill patients and the intracellular HSP content of muscle will correlate with the amount of glutamine given.

CHAPTER 2

MATERIALS AND METHODS

2.1. Model systems

2.1.1. Basic culture techniques of muscle cells

C2C12 cells were purchased derived from an existing mouse myoblast cell line (Yaffe and Saxel. 1977). C2C12 cells were cultured in 6-well plates at 37°C in 5% CO_2 in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, Dorset, UK) containing 0.45% (w/v) glucose with 2mM glutamine, 50 I.U./ml penicillin, 50μg/ ml streptomycin and 12% foetal calf serum. Cells were cultured until 80% confluent; media was changed on alternate days throughout the experiment. When cells reached 80% confluence, media was changed to DMEM with 2mM glutamine, 50 I.U./ml penicillin, 50μg/ ml streptomycin (Invitrogen, UK) and 2% horse serum (Invitrogen, UK) to differentiate the cells into myotubes as previously described by Maglara et al (2003). Cells were harvested on day 9. Myotubes was assessed by light microscopy (Zeiss axiovert microscope, Carl Zeiss, Germany) at every media change (48hours). Representative light microscopy photographs were obtained on day 9 of differentiation, after which the myotubes were harvested in Dulbecco's- phosphate buffered saline (D-PBS, Sigma-Aldrich, Dorset, UK).

The cells were centrifuged (Eppendorf centrifuge 5417R, Germany) at 14,000g for 5mins. The cell pellets were re-suspended in 1% sodium dodecyl sulphate (SDS) containing a range of protease inhibitors (2.4.1) and sonicated on ice.

2.1.2. Clinical trial

2.1.2.1. Patient selection

2.1.2.1.1. Recruitment

All admissions to the Whiston hospital Intensive Care Unit and the Royal Liverpool University hospital Intensive Care Unit were screened for participation between June 2006 – June 2007. To be able to participate the

following inclusion criteria needed to be fulfilled. Further, all exclusion criteria needed to be absent.

2.1.2.1.2. Inclusion

- All admissions to intensive care less than 24 hours from admission with an
  Acute Physiology and Chronic Health Evaluation II score (APACHE II) >10
- Proficient in reading and writing English to be able to understand the information sheet and any other information given.

2.1.2.1.3. Exclusion

- Coagulopathy, defined as
  1. International normalized ratio (INR) > 1.7
  2. Platelet count < 100 x 10^9/L
- Bleeding disorder
- Hepatic impairment defined as a total bilirubin concentration of more than 45 micromol/l or a more than fivefold increase of Alanine aminotransferase (ALT) above normal
- <18 years of age
- Pregnancy
- Expected to be discharged within 48hours from admission as judged by the Consultant in charge at the day of screening for participation
- Expected to die within 48hours from admission as judged by the Consultant in charge at the day of screening for participation
- Malignant disease if not surgically resected.
- Unable to perform biopsy on clinical grounds
- On total parenteral nutrition

2.1.2.1.4. Consent

The patients was entered into the study if they or their relatives on the patients behalf (should the patient be unable to give informed consent) agreed to take part. The study was explained, an information sheet was handed out and in the case of agreement the patient or relative was asked to sign a consent/assent form.

Retrospective consent was obtained as soon as possible in cases where relatives or next of kin signed an assent form.

2.1.2.2. Trial design

2.1.2.2.1. Randomisation

On recruitment the patients were block randomised for age (> 65 years) by the Whiston hospital pharmacy department to receive either glutamine (0.5g per kg bodyweight) or iso-caloric glucose.

2.1.2.2.2. Investigations

2.1.2.2.2.1. Muscle biopsy

Muscle biopsies were taken from the *vastus lateralis of the thigh* under local anesthesia (1% Lidocaine) using a Pro-Mag 2.2 biopsy gun (MD-TECH, Manan Medical Products, Northbrook, IL). Samples obtained (~50 mg) were immediately frozen in liquid nitrogen and stored at –80°C for later analysis. Once the biopsy was completed the wound was cleaned, closed with steri-strips and covered with an adhesive dressing (see Figure 2.1).

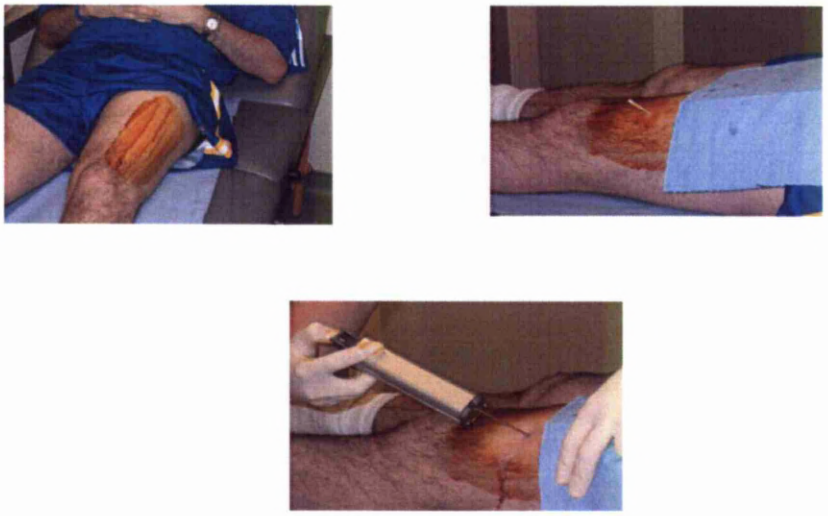


Figure 2.1 Demonstration of muscle biopsy

A second and third biopsy were taken at 48 +/- 4hours post randomisation and at 96 +/- 4 hour post randomisation using alternate legs for each biopsy (same procedure as above). Coagulation status was checked before each biopsy. Biopsies was not performed when

- International normalised ratio (INR) > 1.5
- Platelet count < 100 x 10^9/l

2.1.2.2.2.2. Blood samples

Blood samples were obtained before, at 48 and 96 hours after randomisation from an indwelling arterial catheter (standard device for patients on ITU) for plasma (Na EDTA tubes, S-Monovette, Nuembrecht, Germany) and serum (S-Monovette Serum Gel S, Nuembrecht, Germany), centrifuged at 1500g for 5min. Serum and plasma was stored at -80^{0}C.

2.1.2.2.3. Glutamine dosing

Glutamine (0.5g per kg bodyweight) was chosen in our study which resembles a large glutamine dose with no significant side effects in an alanine-glutamine dipeptide form (Tjaeder et al. 2004) The intravenous infusion was administered as early as possible from randomisation (~1 hour) over ~ 4 hours.

2.1.2.2.4. Trial size

This clinical study was a pilot study. An exact power calculation was not possible as the primary endpoint was not tested in relationship to glutamine in humans before in the manner we proposed. Therefore the aim was to recruit 30 participants, 15 in each group (Lancaster. 2004).

2.1.2.2.5. Flow chart describing patient study

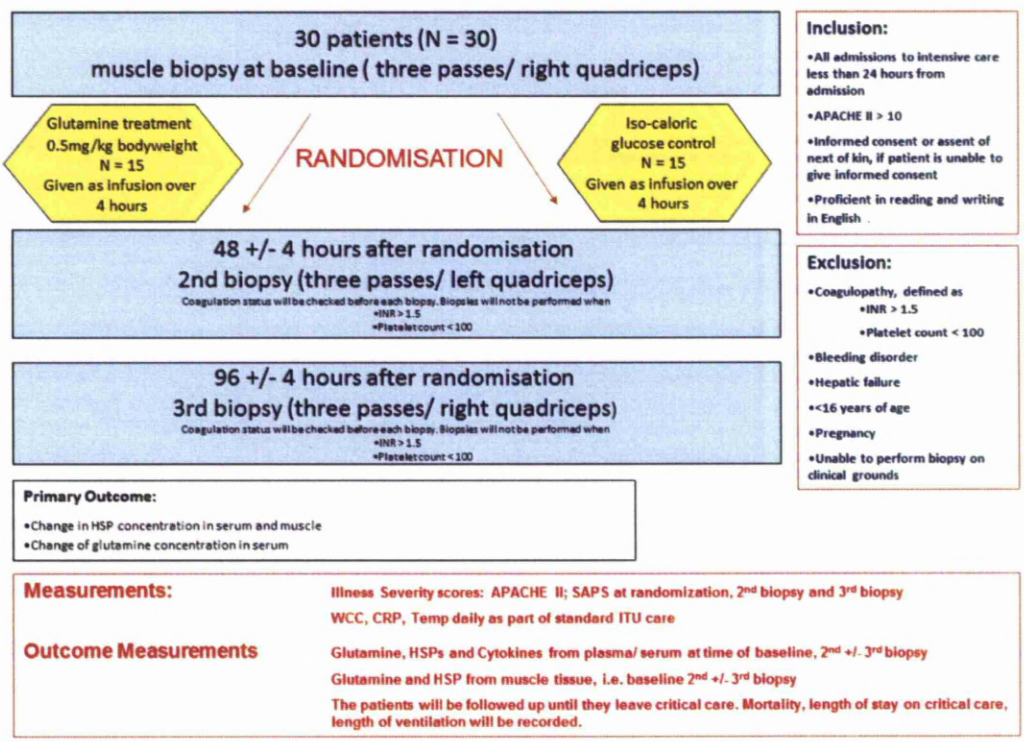


Figure 2.2 Flow chart of clinical trial

2.1.2.2.6. Other treatments

All patients received the same standard of care. Any treatment decision was decided by the consultant in charge and was completely independent of the clinical trial.

2.1.3. Outcomes

2.1.3.1. Primary outcomes:

- Change in HSP concentration in serum or muscle
- Change of Interleukins TNF-α, IL 1β, IL 6 and IL 10
- Change of glutamine concentration in plasma
- Change of total and oxidised glutathione in plasma and muscle

2.1.3.2. Secondary outcomes:

- Change of Severity Scores (APACHE II, Simplified Acute Physiology Score (SAPS), Sequential Organ Failure Assessment Score (SOFA)
- Change in white cell count (WCC), C-reactive Protein (CRP), temperature
- Length of ventilation
- Length of critical care stay
- Critical care mortality

2.1.3.3. Measurements

Illness severity scores (APACHE II, SOFA and SAPS) were assessed at randomization, at 48hours and 96hours post randomisation. WCC, CRP, Temp were recorded daily as part of standard ITU care.

2.1.3.4. Outcome measures

Glutamine, HSPs and cytokines were measured from blood taken at the same time as the baseline, 2nd and 3rd muscle biopsy.
Glutamine and HSP were measured from muscle tissue taken at baseline, 2nd and 3rd biopsy. The patients were followed up until they left critical care.

2.1.4. Ethical approval

The Research Ethics Committee gave ethical approval for the research study, reference number: REC 06/Q1502/43.
The European Drug Regulation Authorities Clinical Trial gave approval, reference number: EudraCT: 2006-000576-33 [including the Medicines and Healthcare products regulatory agency (MHRA)].

2.2. Preparation of samples

2.2.1. Preparation of plasma for glutathione assays

Arterial blood was collected into Na EDTA tubes (S-Monovette, Nuembrecht, Germany) and centrifuged (5min at 1500g). The supernatant was stored at -80°C. 100µl of supernatant was deproteinised by adding 100 µl 1% of sulphosalicylic acid (SSA) to the plasma and centrifuged at 20,000g for 10min at 4^0C (Eppendorf centrifuge 5417R, Hamburg, Germany). The supernatant was used to measure total glutathione content (section 2.3.1) and oxidised glutathione content (2.3.2). The protein-precipitated pellets were used to measure protein thiol content (2.3.3). It was further used to determine the protein content using the Lowry assay (2.3.4).

2.2.2. Preparation of human muscle for glutathione assays

1% sulphosalicylic acid (SSA) was added to muscle powder and homogenised (TRI-R Instruments, Model K43, Rockville Centre, N.Y.). The mixture was centrifuged at 20,000g for 10min at 4^0C (Eppendorf centrifuge 5417R,

Hamburg, Germany). The supernatant was used to measure total glutathione content (section 2.3.1) and oxidised glutathione content (section 2.3.2). The protein-precipitated pellets were used to measure protein thiol content (section 2.3.3). The protein-precipitated pellets were further used to determine the protein content using the Lowry assay (section 2.3.4)

2.2.3. Preparation of samples for SDS-page and western blotting

Reagents

- 0.5 ml Protease inhibitors
  - 1mM Iodoacetimide (50mg)
  - 1mM Benzithonium chloride (112.5mg)
  - 5.7mM Phenylmethylsulphonyl fluoride (PMSF) (250mg) mixed in 25ml of 1% SDS
- 9.5mg EGTA
- 4.5ml 1% SDS

2.2.3.1. Preparation of samples of C2C12 myotubes

C2C12 myotubes (section 2.1.1) were sonicated on ice in the above protease inhibitor solution (section 2.2.3) to avoid protein breakdown prior to analysis and centrifuged at 20,000g for 10 minutes.

2.2.3.2. Preparation of muscle samples

Vastus lateralis muscle was ground in a pestle and mortar under liquid nitrogen. Muscle was homogenized in a 1% solution of sodium dodecyl sulphate (SDS) containing protease inhibitors (section 2.2.3), and centrifuged at 20,000g for 10 minutes.

2.3. Analysis of glutathione and protein thiols

2.3.1. Analysis of total glutathione

Total glutathione was measured using the assay developed by Anderson (1985) which is based on the reaction of glutathione with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB).

Reagents

- 1% (w/v) sulphosalicylic acid (SSA)
- Glutathione reductase (600U/ml; Roche Diagnostics Ltd, East Sussex, U.K.)
- Glutathione (oxidized form, Sigma-Aldrich, Dorset, U.K.)
- Glutathione (reduced form, Sigma-Aldrich, Dorset, U.K.)
- Stock buffer: 143mM sodium phosphate (Na_2HPO_4), pH 7.5
- 6.3mM Tetrasodium EDTA (Na_4EDTA)
- Daily buffer: 0.3mM NADPH (Sigma-Aldrich, Dorset, U.K.) in 14ml stock buffer
- DTNB solution: 6mM 5,5'-dithiobis-(2-nitrobenzoic acid) in 5ml stock buffer

Protocol

Total glutathione content was analysed from supernatant of samples homogenised in 1% (w/v) SSA as described above. A solution containing 16mg of oxidised glutathione and 16mg of reduced glutathione in 10ml of 1% SSA was used to prepare a range of standards between 0.081 and 10.4µM. 1% SSA was used as a blank. A mixture of 75µl of glutathione reductase, 5.25ml daily buffer, 750µl DTNB and 750µl distilled water was prepared. 20µl of standard, sample or blank was placed in a microplate, 200µl of mixture was added. The solution was mixed and the absorbance was measured at 415nm for 10mins in a microplate reader (Powerwave X340, Bio-tech instruments Inc, Vermont, USA.) at a preheated temperature of 37°C.

2.3.2. Analysis of oxidised glutathione

Oxidised glutathione content was determined by selective derivatising of existing reduced GSH by 2-vinyl-pyridine and therefore blocking GSH from being quantified. It further does not interfere with glutathione reductase.

Reagents

- 2-vinyl-pyridine
- Triethanolamine (TEA) 50% in dH_2O
- pH paper

Protocol

Samples were prepared as for total glutathione analysis. 1µl of 2-vinyl-pyridine was added and the sample was vortexed for 1 minute. TEA was added (approximately 1.65µl for standards and 1.45µl for samples) and vortexed for 1 minute. PH was determined (target pH 6-7) and incubated at room temperature for 1 hour. Oxidised glutathione content was determined by the protocol for total glutathione analysis (section 2.3.1).

2.3.3. Analysis of protein sulphydryl group (protein thiol)

The sulphydryl content of muscle protein was determined using the assay by Di Monte et al. 1984)

Reagents

- 0.5M Tris/HCl buffer, pH 7.6
- 1.1mM DTNB in Tris/HCl buffer, pH 7.6
- 1% (w/v) SSA
- 5mM Glutathione (reduced form) in Tris/HCl buffer, (Sigma-Aldrich, Dorset, U.K.)

Protocol

The protein precipitate pellets from muscles homogenised in 1% (w/v) SSA were resuspended in 200µl of Tris/HCl buffer. These were then further diluted 1 in 20 with Tris/HCl buffer. Protein content was determined using the Lowry assay (section 2.3.4). These dilutions were then used for the sulphydryl group assay. A range of standards between 50 and 0.4 µM were prepared from a

5mM solution of reduced glutathione in Tris/HCl buffer, pH 7.6 (Section 2.3.1.). Twenty microlitres of DTNB was added to 200µl of samples and standards, 200µl of Tris/HCl buffer was used as a blank. These were incubated at room temperature for 20mins and then centrifuged (Eppendorf centrifuge 5417R, Germany) at 20,000*g* for 1min. Samples and standards were pipetted onto a 96 well microplate. The absorbance was measured at 415nm using a microplate reader (Powerwave X340, Bio-tech instruments Inc, Vermont, USA.). The sulphydryl group content of samples was calculated using the standard curve.

2.3.4. Lowry assay for determination of protein content

The method by Lowry et al (1951) was used to determine the protein content of samples for protein thiol determination.

Reagents

- 1M KOH
- Bovine serum albumin (BSA) 1mg/ml (Sigma-Aldrich, Dorset, U.K.)
- Solution A: 2% Na_2CO_3 in 0.1M NaOH
- Solution B: 20mM $CuSO_4.5H_2O$ in 1% trisodium citrate
- Solution C: 500µl of Solution B in 25ml of Solution A
- Folin and Ciocalteu's phenol reagent (diluted 1: 1 with distilled water, Sigma-Aldrich, Dorset, U.K.)

Protocol

Samples were diluted 1 in 3 with 1M KOH and incubated at 37°C for 3hrs. A solution of 1mg/ml of bovine serum albumin (BSA) was used to prepare a range of protein standards from 0-250µg/ml. Solution C was prepared immediately before use. Standards and 50µl samples are added to 500 µl of Solution C and then incubated at room temperature for 10mins. 50µl of diluted Folin reagent was added to samples and standards. The mixtures were then vortexed and incubated at room temperature for 30mins. Samples were centrifuged at 20,000g (Eppendorf centrifuge 5417R, Germany) for 1 min and 200µl of samples and standards were added to a microplate. The absorbance

of samples and standards was measured at 750nm using a microplate reader (Powerwave X340, Bio-tech instruments Inc. Vermont, USA). The protein content of the samples was determined using the standard curve.

2.4. Analysis of HSP content by SDS-page and western blotting

2.4.1. Determination of protein content of samples using Bicinchoninic acid (BCA) assay.

The BCA method was used to analyse the protein content of samples. This method is based on the method described by Smith *et al.* (1985)using a commercially available BCA protein assay kit (Sigma Aldrich, Dorset, U.K.).

Reagents

- Bovine serum albumin (BSA) 1mg/ml (Sigma-Aldrich, Dorset, U.K.
- Reagent A: Bicinchoninic acid (BCA) solution, containing: 25mM BCA-Na, 160mM $NaCO_3.H_2O$, 7.0mM Na_2 tartrate, 0.1mM NaOH and 0.95% $NaHCO_3$, pH 11.2 (Sigma-Aldrich, Dorset, U.K.)
- Reagent B: 160mM $CuSO_4.5H_2O$ (Sigma-Aldrich, Dorset, U.K.)
- Reagent C: 25ml of Reagent A and 500µl of Reagent B

Protocol

A range of standards from 25-250µg/ml were prepared from a stock solution of 1mg/ml bovine serum albumin (BSA). 20µl of sample or standard were pipetted in a 96 well microplate. dH_2O was used as blank. Two hundred microlitres of Reagent C was added to the samples, standards and blank, the microplate was incubated a 50°C for 30mins. A stock solution of 1mg/ml of bovine serum albumin (BSA) was used to prepare standards at concentrations of between 25-250µg/ml. The absorbance of samples and standards was measured at 570nm using a microplate reader (Powerwave X340, Bio-tech instruments Inc, Vermont, USA).The protein content of the samples was calculated using the standard curve.

2.4.2. Preparation of samples for SDS-PAGE and western blotting

Reagents

- Laemmli buffer: 46.03 mg/ml SDS, 20.9% glycerol, 2.1% (v/v) β-mercaptoethanol, 0.052 mg/ml bromophenol blue in 0.128M Tris/HCl buffer, pH 6.8.

Protocol

After the determination of the protein content of the individual samples using the BCA method, 100µg of total protein was added to an equal volume of Laemmli buffer. The positive control was prepared from 0.25µg HSP 70 (Stressgen Inc, Canada), 0,5µg HSC 70 (Stressgen Inc, Canada), 0.25µg HSP 60 (Stressgen Inc, Canada) and 0.05µg HSP 25 (Stressgen Inc, Canada) and an equal volume of Laemmli buffer. Samples and positive control were loaded onto a polyacrylamide gradient gel alongside a molecular weight rainbow marker (Amersham Biosciences, U.K.), after 5 min of boiling and subsequent passive cooling to room temperature.

2.4.3. Preparation of polyacrylamide gradient gels

Reagents

- Stock acrylamide solution: 30% acrylamide, 0.8% bisacrylamide cross-link, in dH_2O (Protogel, National Diagnostics, USA)
- Gel buffer: 1.5M Tris/HCl, 0.384% SDS, pH 8.8. (Protogel buffer, National Diagnostics, USA)
- Stacking buffer: 0.5M Tris/HCl, 0.4% SDS, pH 6.8. (Protogel stacking buffer, National Diagnostics, USA)
- 12% acrylamide solution (100ml): 40ml stock acrylamide solution, 26ml gel buffer, 32.9ml dH_2O
- 4% stacking gel solution (100ml): 13ml stock acrylamide solution, 25ml stacking buffer, 61ml dH_2O

- 10% (w/v) Ammonium persulphate (APS)
- NNN'N'-tetramethylethylene-diamine (TEMED)

Protocol

100µl of 10% APS, 10ml of 12% acrylamide solution (see above) and 10µl of TEMED were mixed and poured into a glass cavity of 8cm x 10cm x 2mm made up by two glass plates and a spacer. The mixture was allowed to polymerize. The stacking gel was prepared as follows. 100µl of 10% APS was mixes with 10ml of 4% acrylamide solution (see above). 25µl TEMED was added. A well comb was positioned to facilitate well formation. The stacking gel was then poured on top of the 12% gel to create a stacking gel of about 1 - 1.5 cm thickness.

2.4.4. Electrophoresis of proteins

Reagents

- Electrophoresis buffer: 10X Tris/Glycine/SDS (0.025M Tris, 0.192M glycine, 0.1% (w/v) SDS; National Diagnostics, USA)

Protocol

Anachem Electrophoresis tank with 10X electrophoresis buffer was used for electrophoresis of samples. A constant current of 20mA per gel was applied until samples had run through the 4% stacking gel. The current was increased to 40mA per gel until the rainbow marker had reached the bottom of the gel. The tank was cooled with H_2O during electrophoresis.

2.4.5. Western blotting of separated proteins

Reagents

- Anode 1 buffer: 0.3M Tris in a 20% methanol solution, pH 10.4.
- Anode 2 buffer: 25mM Tris in a 20% methanol solution, pH 10.4.
- Cathode buffer: 40mM 6-amino n hexanoic acid in a 20% methanol solution, pH 7.6.

Protocol

To transfer proteins to a nitrocellulose membrane the Multiphor II discontinuous blotting system (Pharmacia, Milton Keynes, U.K.) was employed. This system comprised of two graphite plate electrodes. The 12 % acrylamide gel was, after removal of the stacking gel, placed on top of a nitrocellulose membrane and sandwiched between the electrodes with Whatman No 1 filter paper pre-soaked in anode and cathode buffers as shown below (Figure 2.3). A constant current of 0.8mA/cm^2 was applied to the system for 1.5hrs.

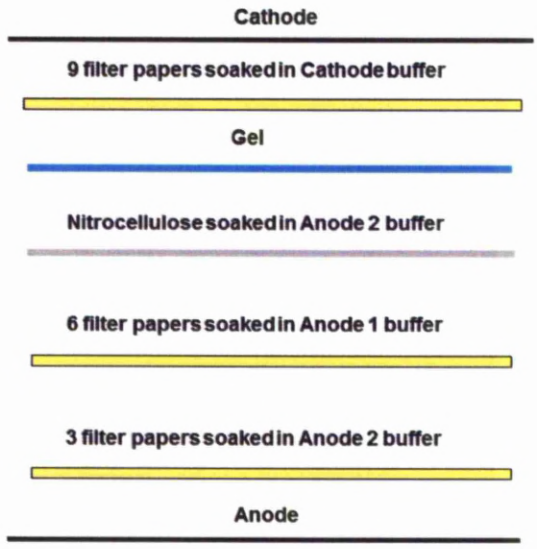


Figure 2.3 Schematic diagram of western blotting

2.4.6. Probing of nitrocellulose membrane for HSP content

Reagents

- PBS solution: 0.05M KH_2PO_4, 0.05M Na_2HPO_4, 1.3M NaCl in dH_2O, pH 7.2.
- PBS solution: 0.05M KH_2PO_4, 0.05M Na_2HPO_4, 1.3M NaCl in dH_2O, pH 6.0.
- PBS/Tween solution: 0.05% (v/v) polyoxyethylene-sorbitan monolaurate (Tween 20) in PBS solution.
- Blocking solution: 5% (w/v) powdered milk in PBS/Tween

Protocol

After transferring the proteins onto the nitrocellulose membrane, the nitrocellulose membrane was placed in 50ml of a blocking solution for either 1 hour at room temperature or overnight at 4°C. The membrane was then washed in PBS/Tween solution and analysed for HSP 60, HSC 70, HSP 70 and HSP 25 using a range of monoclonal or polyclonal antibodies. The nitrocellulose membrane was exposed for an hour in 10ml of a solution of PBS/Tween containing the primary antibody at the concentration outlined (section 2.4.7.) on a rocking table. Following exposure to the primary antibody, the membrane was washed 3 x 10 min in PBS/Tween solution. The nitrocellulose membrane was then exposed for 1 hour at room temperature to the relevant peroxidase labelled secondary antibody in PBS/Tween. 25% Fetal calf serum (FCS) was used as a non-specific blocking agent. The membrane was then washed as before (3x10mins in PBS/Tween) and placed in PBS pH6.0 for 15min (optimal pH for the peroxidase activity). The membrane was developed using SuperSignal West Dura chemiluminescent detection kit (Pierce, Rockford, USA). The membrane was placed between acetate sheets and analysed using a Bio-Rad Chemi-XRS System and QuantityOne software (Bio-Rad, Hercules, USA).

2.4.7. Primary Antibodies used

HSP 70 (Stressgen Inc, Canada, SPA-810). Mouse monoclonal antibody specific against the inducible HSP 70 (HSP 72). A 1:1000 dilution was used.

HSC 70 (Stressgen Inc, Canada, SPA-815). Rat monoclonal antibody specific against the constitutively expressed HSC 70 (HSP 73). A 1:1000 dilution was used.

HSP 60 (Sigma-Aldrich, U.K, 383-491.). Mouse monoclonal antibody against the human HSP 60 amino acids. A 1:500 dilution was used.

HSP 25 (Stressgen Inc, Canada, SPA-801). Rabbit polyclonal antibody against the phosphorylated and non-phosphorylated forms of HSP 25. A 1:2500 dilution was used.

HSP 10 (Stressgen Inc, Canada, SPA-110). Rabbit antibody to the sequence near the C-terminus of HSP 10. A dilution of 1:2000 was used.

2.4.8. Secondary Antibodies

Anti-Mouse (Sigma, Dorset, U.K. Catalogue number A-2554)
Peroxidase conjugated goat anti-mouse IgG (FC specific). A 1:1000 dilution was used.

Anti-Rat (Sigma, Dorset, U.K. Catalogue number A-5795).
Peroxidase conjugated rabbit anti-rat IgG. A 1:1000 dilution was used.

Anti-Rabbit (Sigma, Dorset, U.K. Catalogue number A-0545).
Peroxidase conjugated goat anti-rabbit IgG. A 1:1000 dilution was used.

2.4.9. Removing antibodies and re-probing the nitrocellulose membrane

Reagents

- 100mM β-mercaptoethanol (Sigma-Aldrich, Dorset, U.K.)
- 2% (w/v) SDS
- 62.5mM Tris/HCl, pH 6.7
- PBS/Tween solution, pH 7.2
- Blocking solution containing 5% (w/v) powdered milk PBS/Tween

Protocol

After exposure to chemiluminescent detection solution (Pierce, Rockford, USA), the membrane was washed in PBS/Tween solution for 5mins. To remove previously used antibodies, the nitrocellulose membrane was incubated for 30mins at 50°C in a solution containing the following ingredience: 100mM β-mercaptoethanol, 2% SDS, 62.5mM Tris/HCl, pH 6.7. The membrane was then washed for 2x10mins in PBS/Tween solution at room temperature and then placed in 50ml of blocking solution for 1hr at room temperature or alternatively at 4°C overnight to be ready to be analysed for further HSPs.

2.5. Enzyme Linked Immuno Sorbent Assay (ELISA)

2.5.1. Enzyme Linked Immuno Sorbent Assay (ELISA) to determine cytokine concentrations in serum.

Commercially available solid phase sandwich ELISA kits to determine the concentrations of Tumour Necrosis Factor α (TNF-α) in serum (Human TNF-α, Invitrogen, CA – KHC3011), Interleukin 1beta (IL 1b) in serum (Human IL1b, Invitrogen, CA –KHC 0011) Interleukin 6 (IL 6) in serum (Human IL 6, Invitrogen, CA – KHC0061) and Interleukin 10 (IL 10) in serum (Human IL10, Invitrogen, CA – KHC0101) were used. ELISAs were carried out as per manufacturers' instructions.

Reagents

- Used as per manufacturers recommendation. Included in commercially available ELISA kits

Protocol – principle of method (manufacturer's information)

The used ELISA kits were solid phase sandwich ELISA kits. A monoclonal antibody specific for the particular cytokine in question was coated onto the micro plate wells. All samples were pipetted into the wells including standards. The antigen bound to the antibody were pre-fixed onto the microplate well. After washing, a second biotinylated antibody against the same cytokine was added to bind to the same antibody complex. After washing and removal of excess 2^{nd} antibody Streptavidin-Peroxidase (enzyme) was added. This bound to the biotinylated 2^{nd} antibody. After further incubation and washing a substrate solution was added. This solution produces colour when in contact with Streptavidin-Peroxidase. The intensity of the colour is proportional to the concentration of the particular cytokine.

The absorbance of samples and standards was measured at 450nm using a microplate reader (Powerwave X340, Bio-tech instruments Inc, Vermont, USA.).The protein content of the samples was calculated using the standard curve.

2.5.2. Enzyme Linked Immuno Sorbent Assay (ELISA) to determine Cytokine concentrations in cell culture medium

Solid phase sandwich ELISA kits were used to determine the concentrations of TNF-α, IL 1b and IL 6 in cell culture medium (Mouse TNF-α, Invitrogen, CA – KMC3011), (Mouse IL 1b Invitrogen, CA – KMC0011), (Mouse IL 6 Invitrogen, CA – KMC0061). ELISAs were carried out as per manufacturers' instructions.

Reagents

- Used as per manufacturers recommendation. Included in commercially available ELISA kits

Protocol

Identical protocol used in section 2.5.1.

The absorbance of samples and standards was measured at 450nm using a microplate reader (Powerwave X340, Bio-tech instruments Inc, Vermont, USA.).The protein content of the samples was calculated using the standard curve.

2.5.3. Enzyme Linked Immuno Sorbent Assay (ELISA) to determine Heat Shock Protein 70 (HSP 70) concentrations in serum

Solid phase sandwich ELISA kits were used to determine the concentrations of HSP 70 in serum. ELISAs were carried out as per manufacturers' instructions.

Reagents

- Used as per manufacturers recommendation. Included in commercially available ELISA kit

Protocol

Principle of method (manufacturer's information)

Similar protocol to protocol used in section 2.5.1.

The absorbance of samples and standards was measured at 450nm using a microplate reader (Powerwave X340, Bio-tech instruments Inc, Vermont, USA).The protein content of the samples was calculated using the standard curve.

2.6. High Performance Liquid Chromatography (HPLC) to determine amino acid content of plasma samples

Regents

- 35% sulfosalicylic acid (SSA)
- 75mM sodium acetate pH 5.58 containing 2.5% of acetonitrile
- 70%v/v acetonitrile/water (ultrapure), 32mM sodium acetate pH 6.2

Protocol

A mixture of components is flushed through a chromatography column. Separation of the different compounds is achieved by the different affinities of the individual compounds to a stationary phase in the column. The smaller the affinity a molecule has for the stationary phase, the shorter the time spent in a column. These differential rates of migration as the mixture moves over adsorptive materials provide separation (Carrier and Bordonaro.1994) Samples were treated with 20µl of 35% sulfosalicylic acid for each 200µl of sample to precipitate the protein. The solution was vortexed and left at room temperature for 20 minutes. The samples were then centrifuged, the supernatant separated and 10µl was loaded onto the analyser.

Solvent A: 75mM sodium acetate pH 5.58 containing 2.5% of acetonitrile

Solvent B: 70%v/v acetonitrile/water (ultrapure), 32mM sodium acetate pH 6.2

Column: 2.1x25mm ODS PTC column. Flow rate: 310µl/min;

Column temperature: 31^0C

The ABI 420a amino acid analyser at a wavelength of 254nm was used (Heidelberg, Germany). The results were analysed with Dionex Chromeleon v4.30 software (Camberley, Surrey, U.K.) (Linse KD et al, 1997)

CHAPTER 3

EFFECT OF MODIFIED EXTRACELLULAR GLUTAMINE CONCENTRATION ON HSP CONTENT OF MUSCLE CELLS DURING DIFFERENTIATION AND FOLLOWING STRESS IN CULTURE

3.1. Introduction

3.1.1. Muscle weakness and muscle wasting in critical illness: the role of changes in protein turnover on loss of muscle mass and function

Muscle weakness and muscle wasting is common feature in critical illness (Hasselgren and Fischer. 1998; Latronico and Bolton. 2011). Muscle fibre wasting and atrophy is evident within the first few days of admission to critical care and progresses rapidly. Muscle necrosis however occurs infrequently (Helliwell et al. 1991; Helliwell et al. 1998). Skeletal muscle wasting and muscle weakness is associated with a poor outcome from critical illness. Furthermore, muscle wasting and muscle weakness can cause chronic disability in critical care survivors (Latronico and Bolton. 2011). It is therefore important to abrogate muscle wasting and muscle weakness during critical illness.

It is believed that the catabolism of muscle during sepsis results from a stimulation of proteolysis and an inhibition of protein synthesis (Vary. 1998) although this is a controversial research area and more recently it has been suggested that muscle wasting in sepsis is due to increased protein degradation, were as protein synthesis appears maintained during sepsis (Klaude et al. 2012).

Furthermore, increased levels of circulating cytokines such as TNF-α, which was found to be increased more than twice the normal level in sepsis (Damas et al. 1989), IL 1beta and IL 6 have been implicated in catabolic skeletal muscle protein metabolism, which occurs during sepsis and SIRS. It has been suggested that these pro-inflammatory cytokines inhibit anabolic hormones and modulate protein synthesis and degradation (Vary. 1998; Lang et al. 2002). It has been shown that this catabolic effect of TNF-α is orchestrated via NF ƙ B activation which is also regulated by endogenous reactive oxygen species and this effect can develop within hours of initiation of sepsis (Li et al. 1998; Li et al. 2003; Li et al. 2000). Chronic activation of NF ƙ B has been implicated in disuse atrophy and contractile dysfunction during mechanical ventilation (Smuder et al. 2012).

3.1.2. Protective effect of Heat Shock Proteins

The expression of stress or heat shock proteins (HSPs) is one of the most highly conserved mechanisms of cellular protection and may be central to protect against the assault from systemic inflammation as seen during critical illness. Heat shock proteins (HSPs) are molecular chaperones that are rapidly synthesized within the cell following a variety of stresses such as oxidative stress, viral infection, hypothermia, heat and TNF alpha (Brooks and Faulkner. 1990; Gething and Sambrook. 1992). Under non-stressful conditions HSPs play an important role in protein-protein interactions such as folding and assisting in the establishment of correct protein conformation and prevention of unwanted protein aggregation (Marimoto et al. 1992). Specifically, to regulate a given activity within the cell, HSPs modulate the equilibrium between an "active" state and an "inactive" state of a given polypeptide. This is achieved by the change of a given protein confirmation, instead of changes in the amount of certain proteins by balancing synthesis (Hinault et al. 2006).

Under conditions of stress, HSPs are required for stabilization of denatured or aggregated proteins (Kiang and Tsokos. 1998; Liu and Steinacker. 2001). All HSPs act to preserve cellular integrity. Cells stressed by a sub lethal insult that induces the expression of HSPs, are rendered more resistant to subsequent extreme stress. One of the possible mechanisms underlying stress-tolerance involves the concept that the proper folding of proteins in a cell requires an intricate set of folding machinery known as molecular chaperones. Thus, when induced following cellular stress, HSPs appear to repair denatured/injured proteins or promote their degradation following irreparable injury (Hightower. 1991)

HSP synthesis is significantly increased in cells after exposure to a wide range of cellular stresses (Kiang and Tsokos. 1998). In 1962 Ritossa first showed a change the appearance of chromosomes after heat shock (Ritossa. 1962) In 1975 Gerner and Schneider showed that non-lethal heat treatment carried a transient protection against subsequent lethal heat stress (Gerner and

Schneider. 1975). Subsequently HSPs have been identified to be synthesised after heat stress (Ashburner and Bonner.1979). The protective effect of increased HSP content has been demonstrated in a number of systems including skeletal muscle whereby prior heat treatment and the resulting increased HSP content provided a significant cytoprotective effect against ROS mediated damage (Maglara et al. 2003). Other researchers have shown a protective effect of heat treatment in different animal sepsis models (caecal perforation; Villar et al. 1994 & LPS endotoxaemia; Chu et al. 1997). In these experiments heat treatment resulted in a significant increase in HSP content of tissue and significant mortality benefit (Villar et al. 1994; Chu et al. 1997)

The protective effect of increased HSP content in the lung was very elegantly shown in a severe sepsis model in Sprague-Dawley rats. Sepsis was induced by the two puncture caecal ligation technique. At the time of operation an adenovirus expressing HSP 70 was administered to the lung via an endotracheal tube. The sepsis-induced lung injury was markedly reduced as was mortality in these animals (Weiss et al. 2002). Another study also showed an inverse correlation between HSP 70 levels in lung tissue and serum cytokines, TNF-α, IL 1β and IL 6 in an animal lung injury model (Vreugdenhil et al. 2003)

It is therefore generally accepted that an increase in the cellular content of HSPs protects tissue against subsequent exposure to damage. Systemic inflammation as seen during critical illness might resemble such damage. An adequate HSP response is therefore believed to be critical for cell survival of various tissues in this context.

3.1.3. Muscle regeneration, repair and HSPs

Skeletal muscle is able to regenerate and repair following damage. This regeneration and repair depends on a reservoir of stem cells known as satelite cells which are present in muscle. When muscle cells are damaged satelite cells are activated and develop into myoblasts. Theses immature muscle cells (myoblasts) proliferate, differentiate and fuse with remaining muscle fibres fascilitating repair (Maglara. 1998; Schultz. 1989).

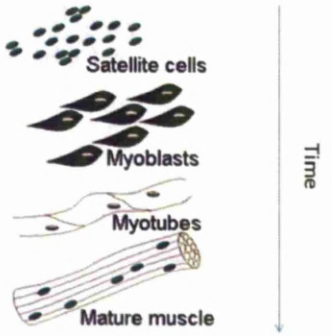


Figure 3.1 Schematic illustration of muscle cell maturation adapted from Komberg. 2013

It is therefore not only important to address strategies to avoid muscle wasting and atrophy but enhance muscle regeneration. It has been suggested that HSPs are required during cellular remodelling and repair. Indeed high HSP content during this remodelling phase has been observed, which is not surprising considering that myoblasts undergo significant remodelling to form mature myotubes with the increased expression of muscle specific proteins (Maglara et al. 2003). As outlined above, muscle wasting and atrophy are common and important phenomenon in critical illness (Latronico and Bolton 2011; Vary 1998). HSP70 content is significantly down-regulated in multiple models of skeletal muscle atrophy (Selsby et al. 2007; Stevenson et al. 2003; Lecker et al. 2004). Maglara et al (1998) demonstrated that proliferation, fusion and maturation of skeletal muscle stem cells or myoblasts were associated with enhanced levels of HSPs. It was further shown that increased HSP concentrations fascilitate regeneration of muscle after muscle damage (McArdle et al. 2004).

3.1.4. Glutamine and HSPs

Glutamine, traditionally considered a non-essential amino acid, now appears to be a conditionally essential nutrient during stress, injury, or illness (Lacey and Wilmore. 1990; Wischmeyer. 2005; see detailed discussion in section 1.3.4). Glutamine supplementation during critical illness has resulted in significant beneficial effects (Novak et al. 2002; Griffith et al. 1997; Singleton et al. 2005b); however mechanisms by which this protection occurs are poorly understood. One mechanism by which glutamine offers protection is thought to be through increased expression of HSPs in a range of tissues. Exposure of monocytes (Eliasen et al. 2006b) and intestinal epithelial cells (Wischmeyer et al. 1997) to physiologically high concentrations of glutamine during heat stress resulted in increased HSP expression and enhanced cell survival. In contrast, exposure of human leukocytes to glutamine concentrations below normal physiological levels attenuated HSP expression (Oehler et al. 2002). Moreover, supra-normal glutamine administration was shown to significantly increase HSP 70 concentration in a rat sepsis model (Maglara 2003). Of note, the glutamine dose correlates with tissue HSP 70 content and importantly facilitated tissue protection in several organs (Musch et al. 2001). This observation generated the hypothesis that glutamine may exert a direct pharmacological effect during sepsis when administered in high doses (Wischmeyer. 2006b).

Important to note is that patients initially present with low glutamine, i.e. deficiencies, particularly in the critically ill (Biolo et al.. 2005) and so the studies described below are aimed both at determining the effect of modified glutamine concentration on muscle HSP content, but also the effect of depletion followed by repletion of glutamine.

3.1.5. Aim of experiments

The hypothesis for this study was that muscle cells treated with low levels of extracellular glutamine will demonstrate modified HSP content and will be unable to respond to stress by an increased content of HSPs. Repletion of

glutamine will reverse this inability to respond to hyperthermia or cytokine-mediated stress and will result in modified cytokine production.

The aims of this study are therefore:

1. To determine the effect of different concentrations of extracellular glutamine on the development, differentiation and maturation of C2C12 myotubes compared with cells in media with the standard 'normal' glutamine concentration of 2mM.
2. To identify the effect of different concentrations of extracellular glutamine on HSP content of untreated C2C12 myotubes or following treatment with hyperthermia or TNF-α compared with cells in media with the standard glutamine concentration of 2mM.
3. To examine the effect of different concentrations of extracellular glutamine on the release of cytokines by C2C12 myotubes following exposure to TNF-α compared with cells in media with the standard glutamine concentration of 2mM.

3.2 Experimental design

3.2.1 The effect of different concentrations of extracellular glutamine on myoblast differentiation and intracellular HSP content

This experiment investigated whether different glutamine concentrations during myoblast differentiation affect the intracellular HSP content of C2C12 cells which is known to be important during regeneration. C2C12 myoblasts were grown in medium as described in Section 2.1.1. When cells reached 80% confluence, media was changed to differentiation medium (DMEM with 2mM glutamine, 50 I.U/ml penicillin, 50μg/ml streptomycin and 2% horse serum) to differentiate the cells into myotubes. After 24 hours of incubation the glutamine concentration was changed to the following concentrations: 0, 0.1mM, 0.25mM, 0.5mM, 1mM, 2mM, 5mM and 10mM glutamine. Media

was changed every 48 hours. At day 9, light microscopy photographs were taken after which cells were harvested as described in Section 2.1.1 and analysed for HSP content as described in Section 2.2.3.1 and 2.4. For schematic illustration of the experimental design see Figure 3.2.

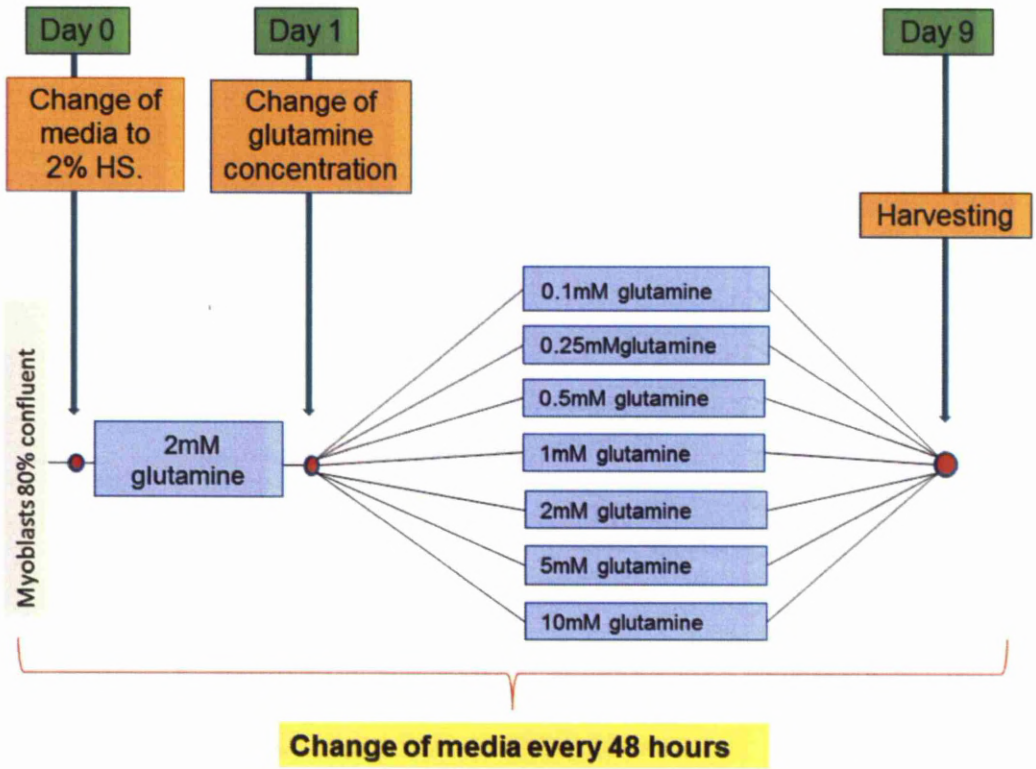


Figure 3.2 Schematic illustration of experimental design. Change of glutamine concentration occurred after 24 hours of differentiation. All other components in the medium were maintained at same concentrations during the experiment. (HS: Horse serum).

3.2.2 The effect of different concentrations of extracellular glutamine on intracellular HSP content after myotube formation – the effect of repletion of glutamine after acute deficiency.

This experiment addressed the question whether 48hours of relatively high (2.5 x normal) glutamine concentration after a period of relative deficiency in

an unstressed cell results in an increased intracellular HSP content compared with normal and relatively deficient (25% of normal) glutamine concentration. To facilitate this, C2C12 myoblasts were cultured in medium as described (Section 2.1.1). At day 4 of differentiation the glutamine concentration in the medium was changed from 2mM to 0.5mM to resemble a modest glutamine deficiency (Biolo et al. 2005). The concentration of 0.5mM glutamine, 25% of normal, was used to facilitate a low glutamine concentration and therefore resemble a relative deficiency as seen in a clinical setting. A similar concentration of 25% of the physiological glutamine concentration has been used by other researchers in a human leucocyte model (Oehler et al. 2002). At day 7 of differentiation the cells were divided into 3 experimental arms of different glutamine concentrations; 0.5mM glutamine, 2mM glutamine and 5mM glutamine. All other components in the medium were maintained at same concentrations as described (Section 2.1.1) and were identical in the three treatment arms. Cells were harvested at day 9 for all experiments (Figure 3.3) to be able to compare the different outcomes in the different experiments and to exclude differences in outcome due to time of harvesting. The time point of harvesting of cells at 48 hours post change of glutamine concentration was chosen as it has been reported that HSP concentrations plateau between 24-48hours post glutamine administration (Wischmeyer et al. 2001). Cells were harvested as described and HSP content analysed as described in Section 2.1.1.

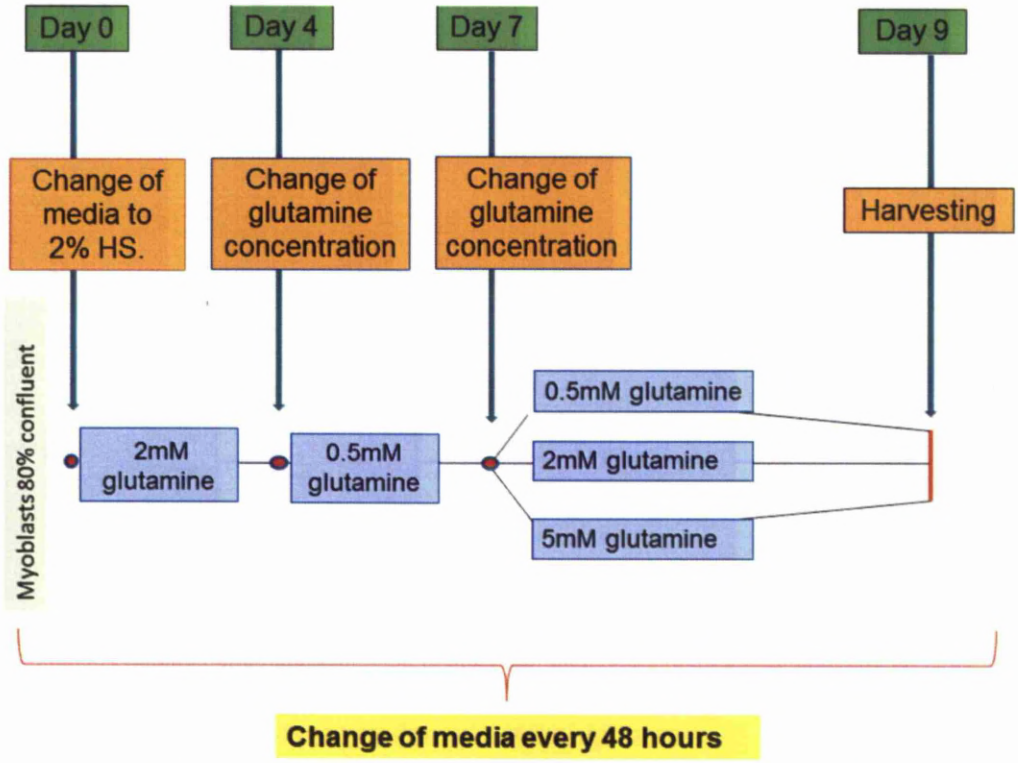


Figure 3.3 Schematic illustration of experimental design. Change of glutamine was as illustrated. All other components in the medium were maintained at same concentrations during the experiment. (HS: Horse serum).

3.2.3. The effect of different concentrations of extracellular glutamine on intracellular HSP content of myotubes following heat stress.

This experiment addressed the question as to whether cells exposed to 48 hours of different extracellular glutamine concentrations demonstrate a different intracellular HSP content following a stress which is known to result in increased HSP synthesis, hyperthermia. The glutamine concentrations used in these studies were chosen to reflect concentrations that were within the physiological ranges identified in patients with sepsis (<10mM) and that did not demonstrate gross changes in cell growth or differentiation (>0.1mM). Thus, the effects of concentrations of 0.5mM glutamine, 2mM glutamine and 5mM glutamine were examined in this study.

This study addressed two important questions:

1) Does a relative glutamine deficiency attenuate the ability of C2C12 myotubes to increase the intracellular HSP content after heat stress?
2) Does a relative high glutamine concentration augment HSP production by C2C12 myotubes after heat stress?

To investigate this, C2C12 myoblasts were grown in medium as described (Section 2.1.1). At day 4 after cells were placed into differentiation medium, the glutamine concentration in the medium was changed from 2mM to 0.5mM to resemble a modest glutamine deficiency (Section 3.2.2). At day 7, the cells were divided into 3 experimental groups of different glutamine concentrations; 0.5mM glutamine, 2mM glutamine and 5mM glutamine. The cells received heat treatment (42^{o}C for 30min) on day 8. The cells were harvested at day 9 (Section 3.2.1) and HSP content was analysed by SDS_PAGE and western blotting (Section 2.4). For schematic illustration of the experimental design see Figure 3.4.

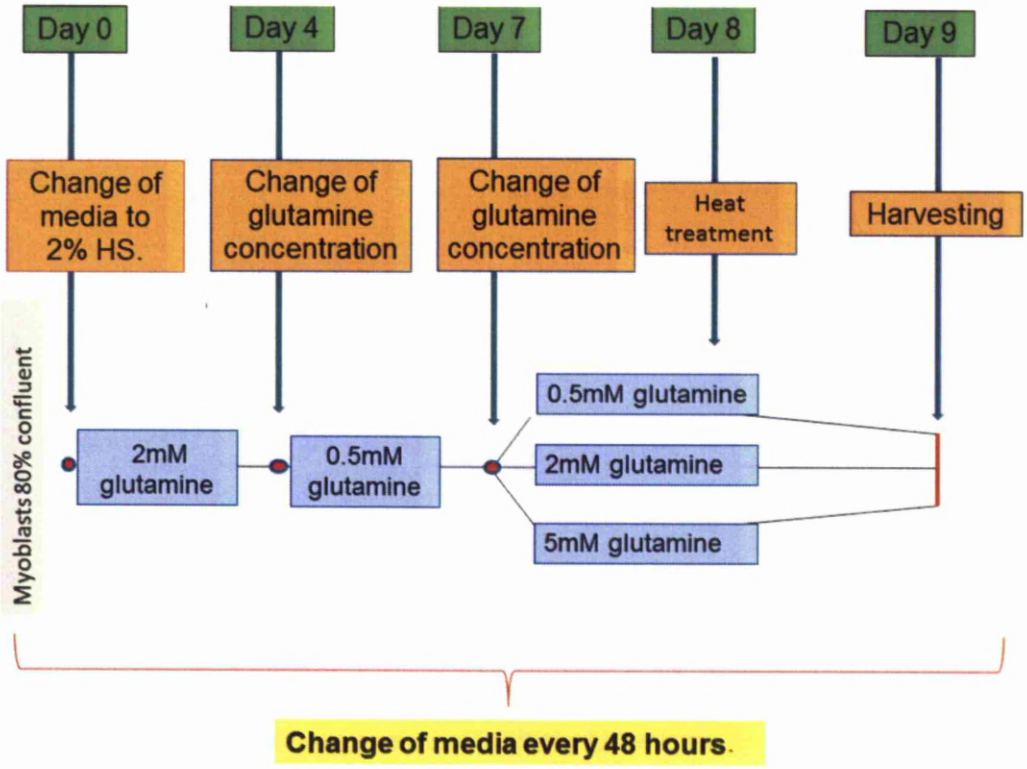


Figure 3.4 Schematic illustration of experimental design. Change of glutamine concentration and heat treatment occurred as illustrated. All other components in the medium were maintained at same concentrations. (HS: Horse serum).

3.2.4 The effect of TNF-α treatment on HSP content and cytokine production of myotubes maintained in 2mM glutamine.

Cytokine production is an integral part of the cell and organism response to infection and inflammation and TNF-α is a major pro-inflammatory cytokine implicated in SIRS seen in critical illness although the effect of modified extracellular glutamine concentrations on the TNF-α induced response of muscle cells is poorly understood.

Initial experiments established:

1) The optimal concentration of TNF-α concentration which resulted in increased HSP content of C2C12 myotubes grown in normal extracellular glutamine concentration.

2) The effect of (1) on cytokine production following TNF-α exposure.

During these experiments, the extracellular glutamine concentration was maintained at 2mM. At day 8, TNF-α (ENZO life science, Exeter, UK) was added to the medium at 4 different concentrations (see Figure 3.4). At day 9 light microscopy photographs were taken after which media and cells were harvested and HSP content was analysed (Section 2.4.). Cytokine concentration in the media was determined using ELISA (Section 2.5.2). For schematic illustration of the experimental design see Figure 3.5.

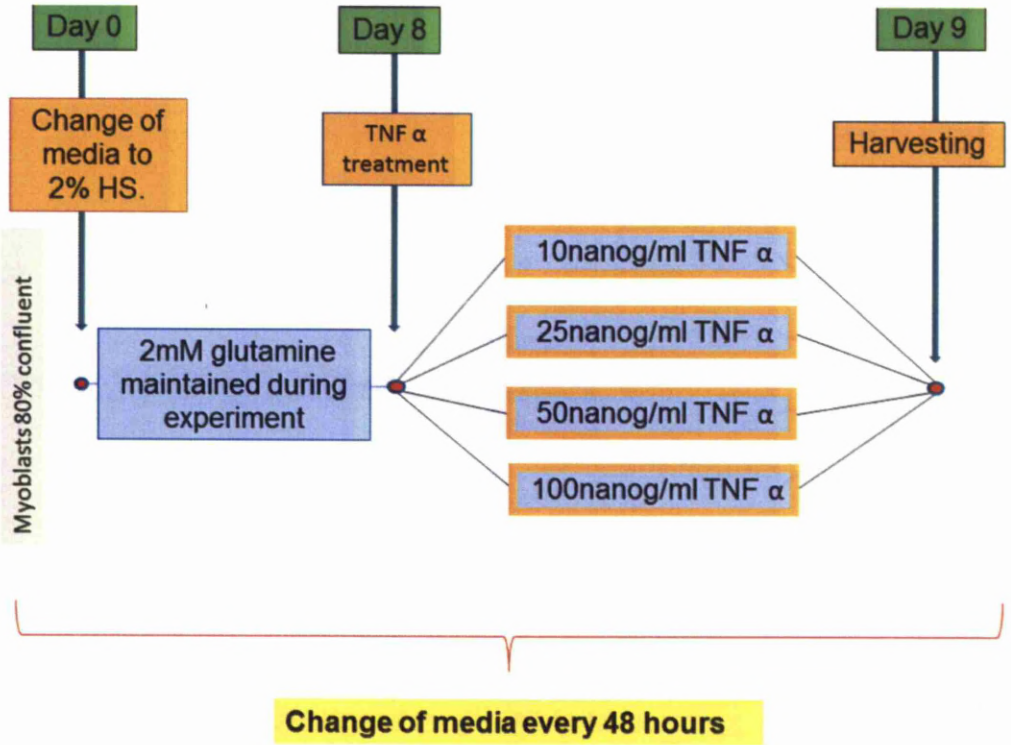


Figure 3.5 Schematic illustration of experimental design. Glutamine concentration of 2mM was maintained throughout the experiment and cells were treated with TNF-α as illustrated. All other components in the medium were maintained at same concentrations during the experiment.

3.2.5 The effect of glutamine deficiency and repletion on intracellular HSP content and cytokine release of C2C12 cells following treatment with TNF-α.

The aim of these experiments were to determine:

1) The effect of prolonged depletion of glutamine following glutamine deficiency on the ability of C2C12 myotubes to increase the intracellular HSP content after treatment with TNF-α (at a concentration known to induce increased HSPs in cells grown at normal glutamine concentration).
2) The effect of a relative high glutamine concentration (2.5 times normal levels) following depletion on the ability of C2C12 myotubes to increase the intracellular HSP content after TNF-α treatment.
3) The effect of prolonged depletion or relative repletion of extracellular glutamine on cytokine release by C2C12 cells following treatment with TNF-α.

To investigate this, C2C12 myoblasts were grown in medium as described (Section 2.1.1) At day 4 of differentiation the glutamine concentration in the medium was changed from 2mM to 0.5mM to resemble a modest glutamine deficiency (Section 3.2.2). At day 7 of differentiation the cells were divided into 3 experimental groups of different glutamine concentrations: 0.5mM glutamine, 2mM glutamine and 5mM glutamine. The cells were treated with 10ng/ml TNF-α on day 8. The cells were harvested at day 9 (Section 3.2.2). For schematic illustration of the experimental design see Figure 3.6. Cells were harvested as described (Section 2.1.1) HSP content was analysed by SDS-PAGE and western blotting (Section 2.4) and cytokine concentrations were measured with ELISA technique (Sections 2.5.2).

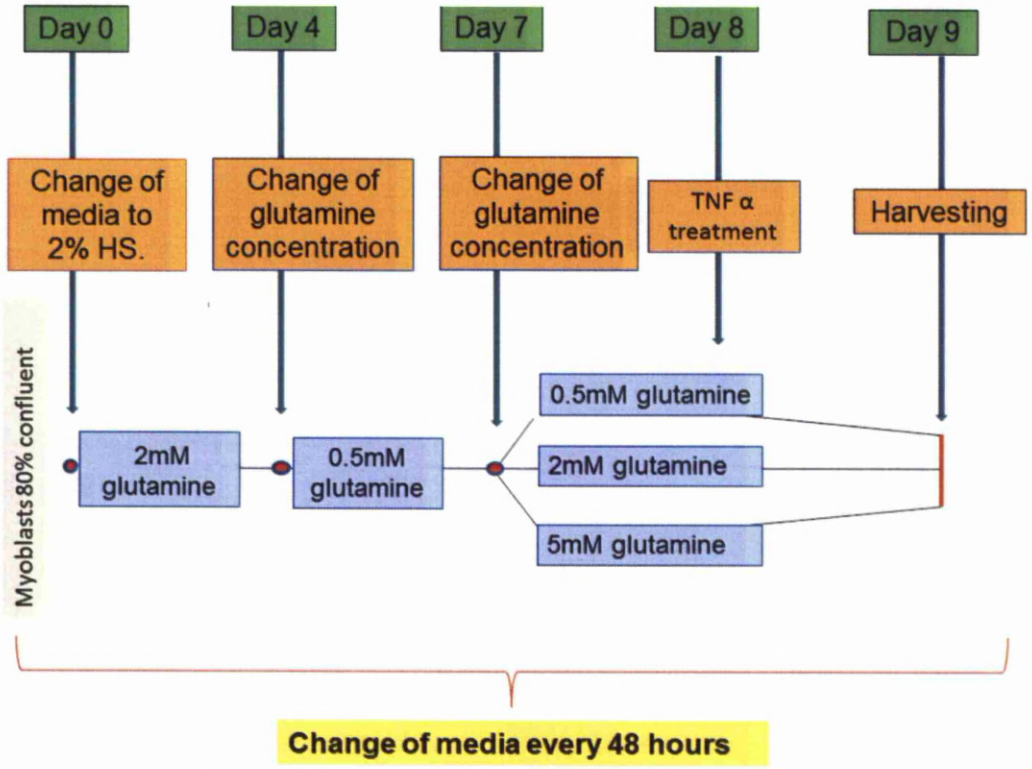


Figure 3.6 Schematic illustration of experimental design. Change of glutamine and exposure to TNF-α was as illustrated. All other components in the medium were maintained at same concentrations during the experiment. (HS: Horse serum).

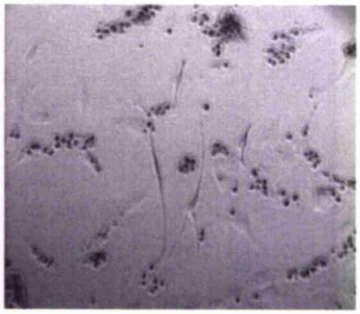
3.2.6. Statistical analysis

Statistical analysis was carried out with Statistical Package for Social Sciences (SPSS) software version 15. Statistical differences were determined using ANOVA, when a significant p-value was observed; post-hoc analysis was performed to identify significance where appropriate. Significance was set at the level of ≤ 0.05. Data are presented as mean ± standard error of the mean (SEM).

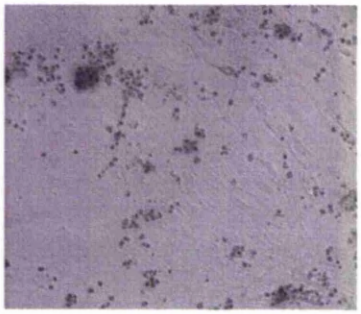
3.3. Results

3.3.1 The effect of different concentrations of extracellular glutamine on myoblast differentiation and intracellular HSP content

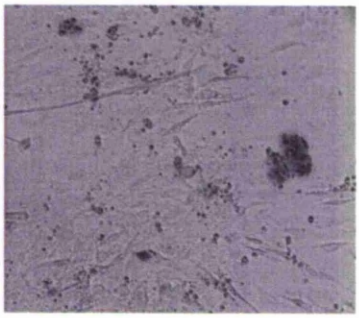
Figure 3.7 shows representative images of the effect of different concentrations of glutamine on the proliferation, fusion and maturation of C2C12 myoblasts. Glutamine concentrations of 0 - 0.5mM in the medium resulted in impaired cell survival and myotube formation upon light microscopy examination compared with cells grown in 2mM glutamine. Myotube formation was not overtly altered at concentrations of 1mM, 5mM and 10mM glutamine in medium.



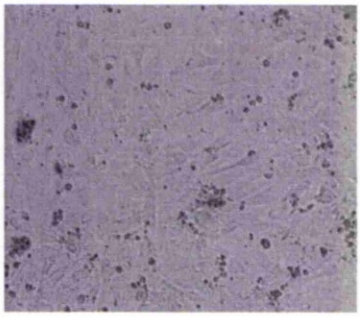
0 mM glutamine



0.1 mM glutamine



0.25 mM glutamine



0.5 mM glutamine

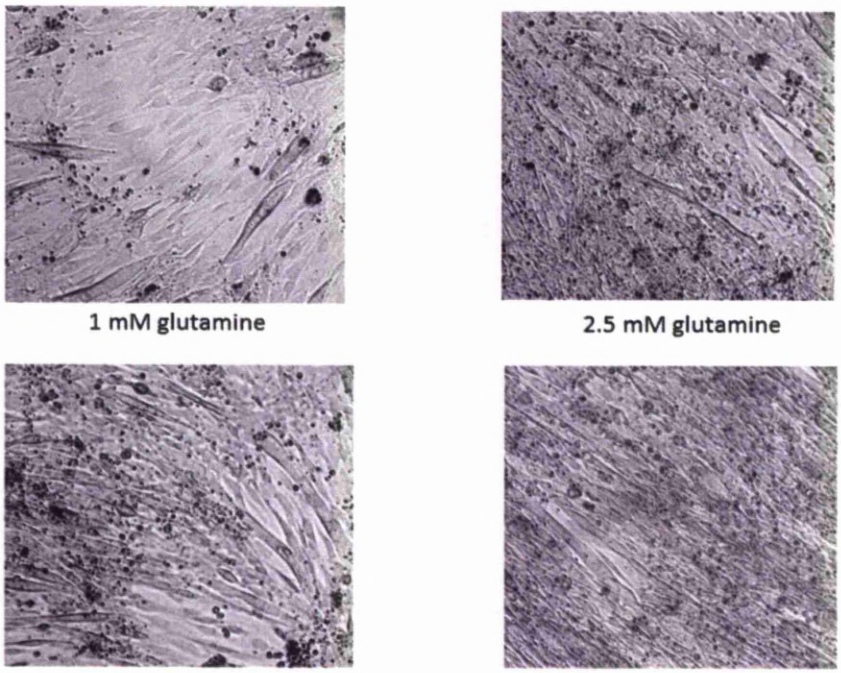


Figure 3.7 The effect of different concentrations of extracellular glutamine on myoblast differentiation at 9 days following change to differentiation media (10x). The glutamine concentration was changed after 24 hours of differentiation to 0; 0.1mM; 0.25mM; 0.5mM; 1mM; 2mM; 5mM and 10mM respectively. All other components in the medium were maintained at same concentrations during the experiment (Section 2.1.1). Media was changed every 48 hours.

Figure 3.8 shows the effect of different concentrations of extracellular glutamine during differentiation of C2C12 myoblasts in cell culture on the intracellular HSP 70 content of cells. Data demonstrated that the HSP 70 content of cells grown in 0.5mM glutamine had a significantly higher content of HSP70 compared with the HSP70 content of cells grown in 2mM, 5mM and 10mM glutamine respectively. Cells grown in 1mM glutamine had a significantly higher content of HSP70 compared with the HSP70 content of cells grown in 5mM and 10mM glutamine.

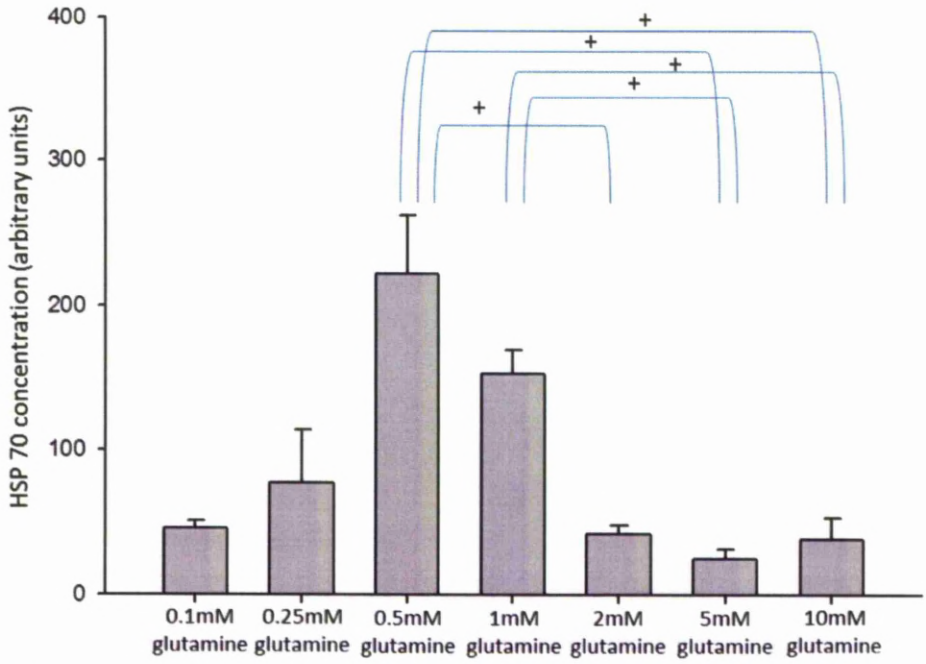


Figure 3.8 The effect of different concentrations of extracellular glutamine on the intracellular HSP 70 content of C2C12 cells during differentiation. Media were changed every 48 hours. Cells were harvested at day 9 of differentiation. HSP 70 concentration expressed in arbitrary units. Data presented as mean +/- SEM, n=5. $^{+}p = < 0.05$.

Following this pilot study, the analyses were restricted to cells treated with 0.1, 1, 2 and 10mM glutamine concentration representing relatively low and high concentrations that resulted in modified HSP content.

Figure 3.9 shows the effect of different glutamine concentrations during differentiation of C2C12 myoblasts in cell culture on the intracellular HSC 70 content. Cells were grown in differentiation media containing 2mM glutamine. The glutamine concentration was changed after 24hours to 0; 0.1mM; 0.25mM; 0.5mM; 1mM; 2mM; 5mM or 10mM respectively. All other components of the medium were maintained at same concentrations during the experiment as outlined (Section 2.1.1). Media was changed every 48 hours. Cells were harvested at 9 days following seeding in differentiation media.

Data demonstrated that the HSC 70 content of cells grown in 10mM glutamine had a significantly higher content of HSC70 compared with the HSC 70 content of cells grown in 2mM. Cells grown in 1mM glutamine and 10mM glutamine had a significantly higher content of HSC70 compared with the HSC70 content of cells grown in 0.1mM glutamine.

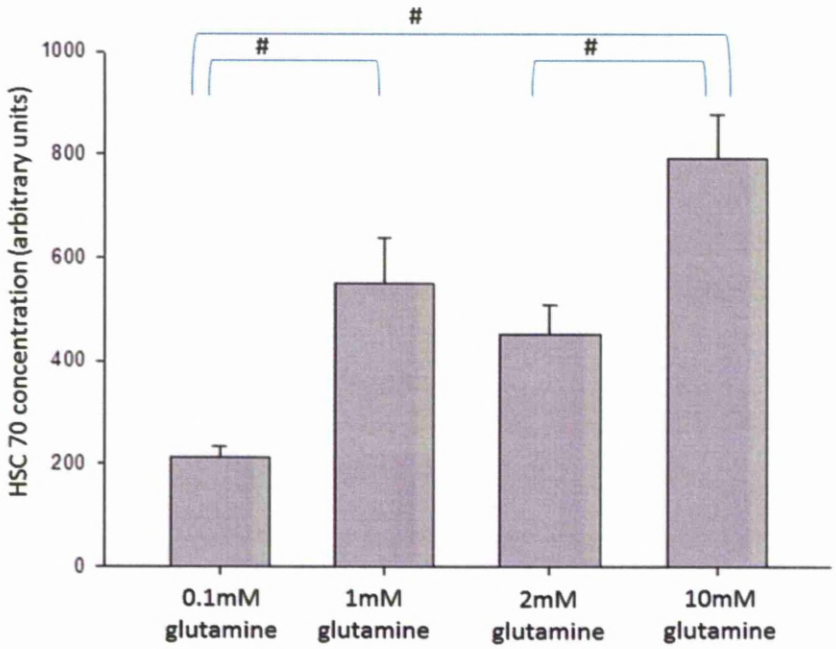


Figure 3.9 The effect of different concentrations of extracellular glutamine on the intracellular HSC 70 content of C2C12 cells during differentiation. Media were changed every 48hours. Cells were harvested at day 9 of differentiation. HSC 70 content expressed in arbitrary units. Data presented as mean +/- SEM, n=4. $^{\#}p = < 0.05$.

Figure 3.10 shows the effect of different glutamine concentrations during differentiation of C2C12 myoblasts in cell culture on the intracellular HSP 60 content. Cells were seeded in differentiation media containing 2mM glutamine. The glutamine concentration was changed after 24hours to 0; 0.1mM; 0.25mM; 0.5mM; 1mM; 2mM; 5mM or 10mM respectively. All other components of the medium were maintained at same concentrations during the experiment as outlined (Section 2.1.1). Media was changed every

48hours. Cells were harvested at 9 days following seeding in differentiation media.

Data demonstrated no significant difference in the HSP 60 content of cells grown in 2mM glutamine compared with the HSP 60 content of cells grown in relatively low or high concentrations of glutamine respectively.

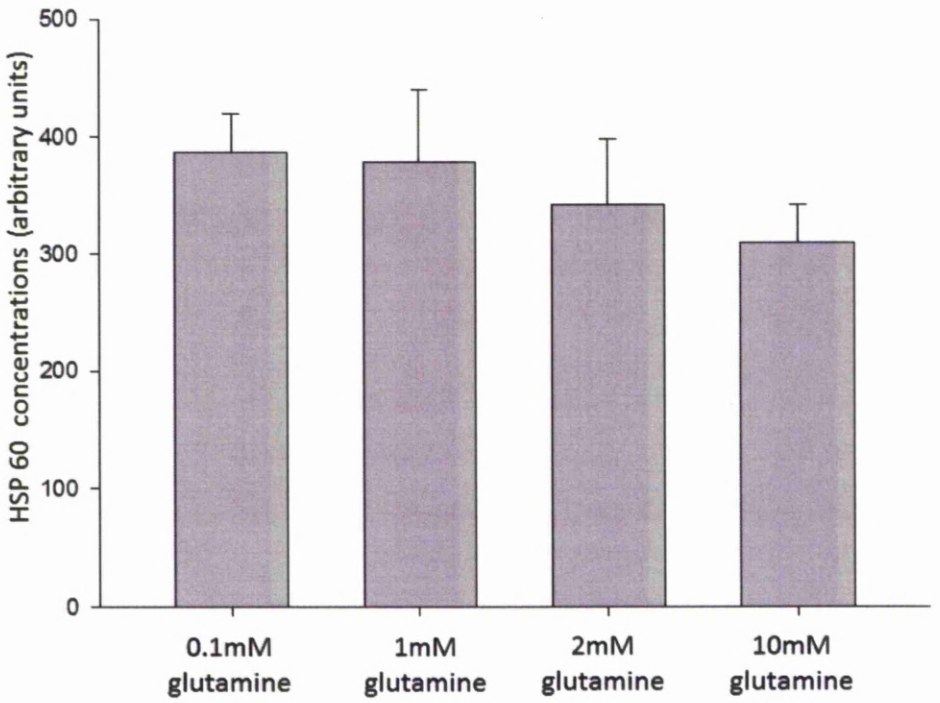


Figure 3.10 The effect of different concentrations of extracellular glutamine on the intracellular HSP 60 content of C2C12 cells during differentiation. Media were changed every 48hours. Cells were harvested at day 9 of differentiation. HSP 60 concentration expressed in arbitrary units. Data presented as mean +/- SEM, n=4.

3.3.2 The effect of different concentrations of extracellular glutamine on intracellular HSP concentration after myotube formation – the effect of repletion of glutamine after acute deficiency.

Figure 3.11 shows HSP 70 content of C2C12 myotubes after growth in relatively deficient glutamine concentrations and repleted with either 0.5mM, 2mM or 5mM glutamine. Cells were seeded in differentiation media containing 2mM glutamine. At day 4 media was changed to media containing 0.5mM glutamine. At day 7 media was changed to media containing either 0.5mM, 2mM or 5mM glutamine and harvested at day 9. Intracellular HSP 70 content was not significantly different between the three conditions at day 9 of the experiment (Figure 3.3.).

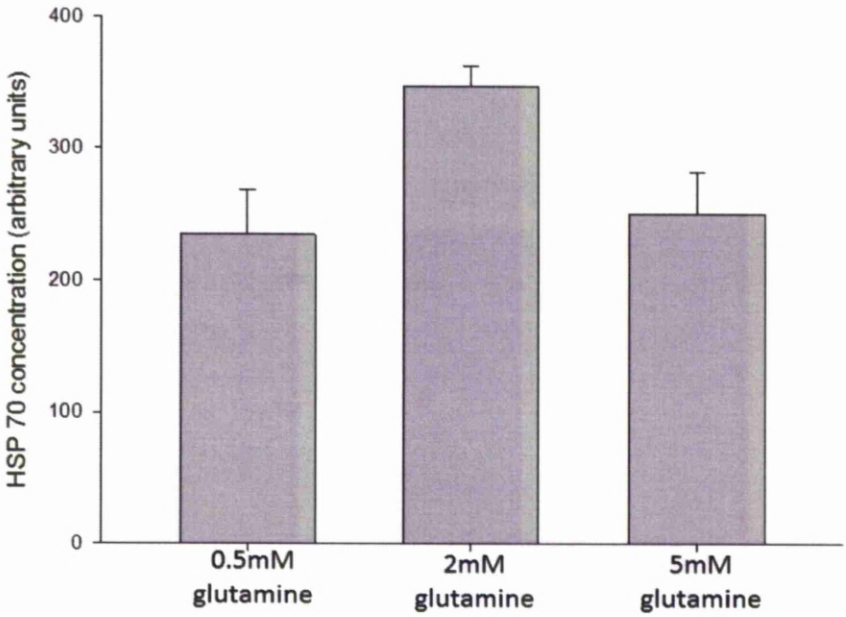


Figure 3.11 HSP 70 content of C2C12 myotubes after growth in relatively deficient glutamine concentrations and repleted with either 0.5mM, 2mM or 5mM glutamine. Intracellular HSP 70 content is expressed in arbitrary

units (as a percentage of positive control). Data presented as mean +/- SEM, n=5.

Figure 3.12 shows HSC 70 content of C2C12 myotubes after growth in relatively deficient glutamine concentrations and repleted with either 0.5mM, 2mM or 5mM glutamine. Intracellular HSC 70 content was not significantly different between the three conditions at day 9 of the experiment (Figure 3.3).

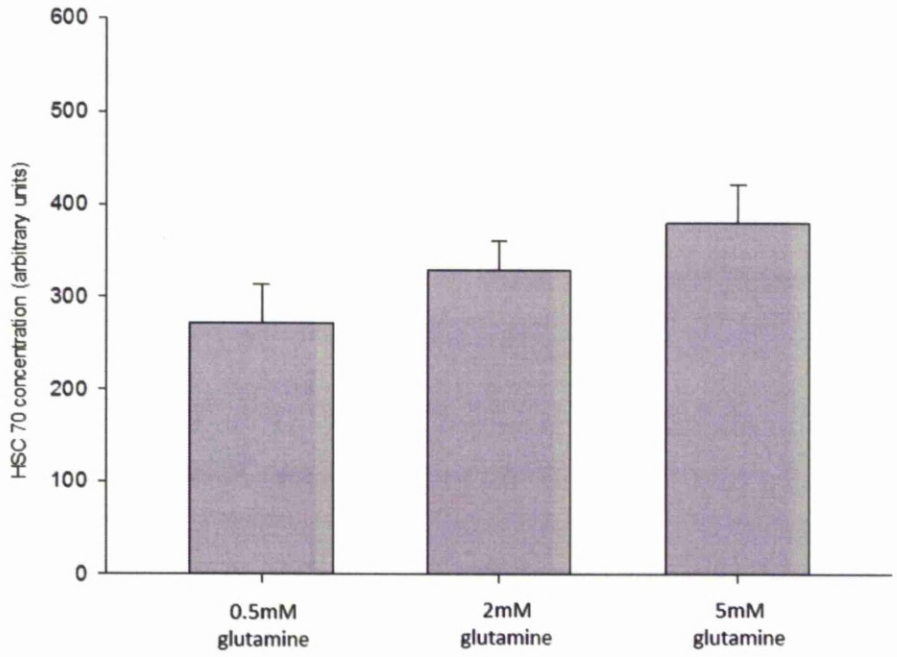


Figure 3.12 HSC 70 content of C2C12 myotubes after growth in relatively deficient glutamine concentrations and repleted with either 0.5mM, 2mM or 5mM glutamine. Intracellular HSC 70 content is expressed as a percentage of positive control. Data presented as mean +/- SEM, n=5.

Figure 3.13 shows HSP 60 content of C2C12 myotubes after growth in relatively deficient glutamine concentrations and repleted with either 0.5mM, 2mM or 5mM glutamine. Intracellular HSP 60 content was not significantly different between the three conditions at day 9 of the experiment (Figure 3.3).

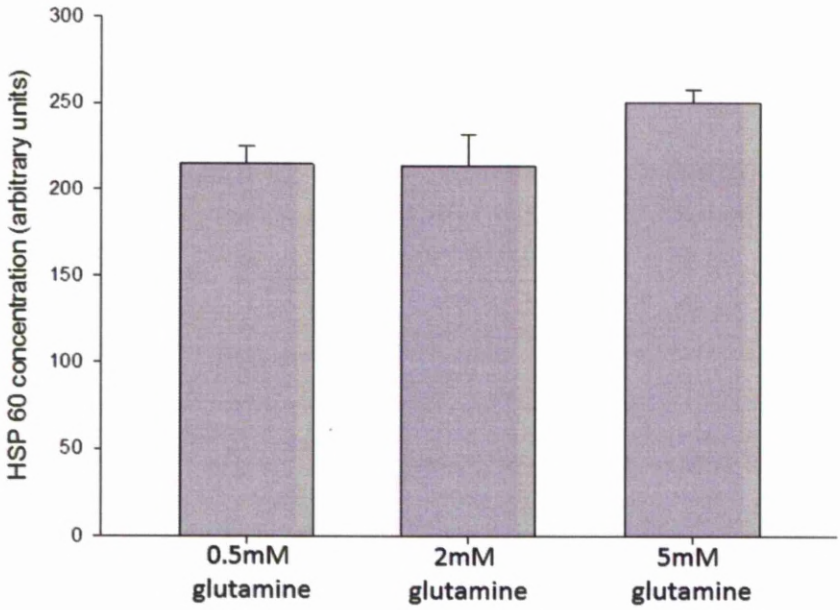


Figure 3.13 HSP 60 content of C2C12 myotubes after seeding (72hours) and grown for 72 hours in 0.5mM glutamine followed by 48 hours in either 0.5mM, 2mM or 5mM glutamine. Intracellular HSC 70 content is expressed in arbitrary units. Data presented as mean +/- SEM, n=5.

3.3.3 The effect of different concentrations of extracellular glutamine on intracellular HSP concentration of myotubes following heat stress.

Initial studies established that treatment of myotubes with a period of hyperthermia at 42°C for 30 minutes resulted in a significant increase in HSP content of cells compared with the HSP content of untreated cells (Figure

3.14). This treatment regime was then used for all further studies and comparisons presented reflect the HSP content of cells maintained in a glutamine deficient media or in a replete media and subjected to hyperthermia compared with cells maintained in 'normal' 2mM glutamine and subjected to hyperthermia.

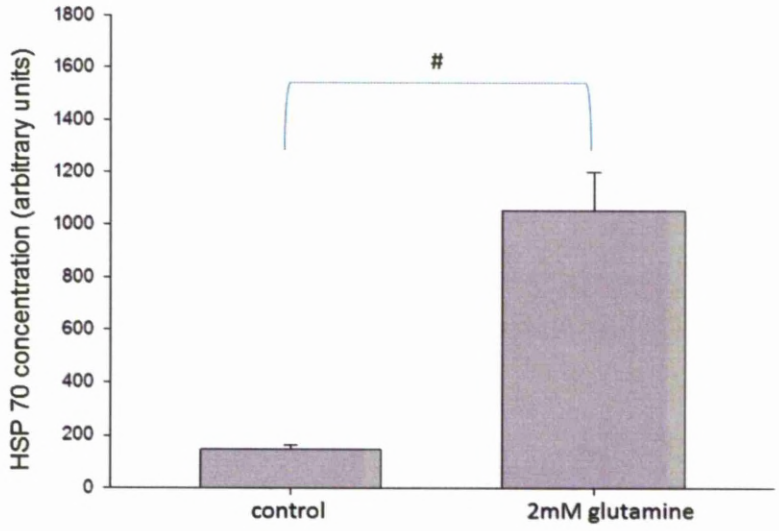


Figure 3.14 HSP 70 content of C2C12 myotubes after subsequent repletion and following heat treatment compared to no heat treatment. Intracellular HSP 70 content is expressed in arbitrary units. Data are presented as mean +/- SEM, n=5. $^{\#}p = < 0.05$.

Figure 3.15 shows HSP 70 content of C2C12 myotubes. Cells were grown in differentiation media containing 2mM glutamine. At day 4 media was changed to media containing 0.5mM glutamine. At day 7 media was changed to media containing either 0.5mM, 2mM or 5mM glutamine. Myotubes were then heat stressed at day 8 and harvested at day 9 (Figure 3.4). The HSP 70 content of heat treated cells grown in 5mM glutamine had a significantly higher content of HSP 70 compared with the HSP 70 content of heat treated cells in 0.5mM glutamine. These data suggest that the hyperthermia-induced HSP70 content of cells was considerably reduced in cells which were maintained in glutamine deficient media and this was corrected with glutamine repletion.

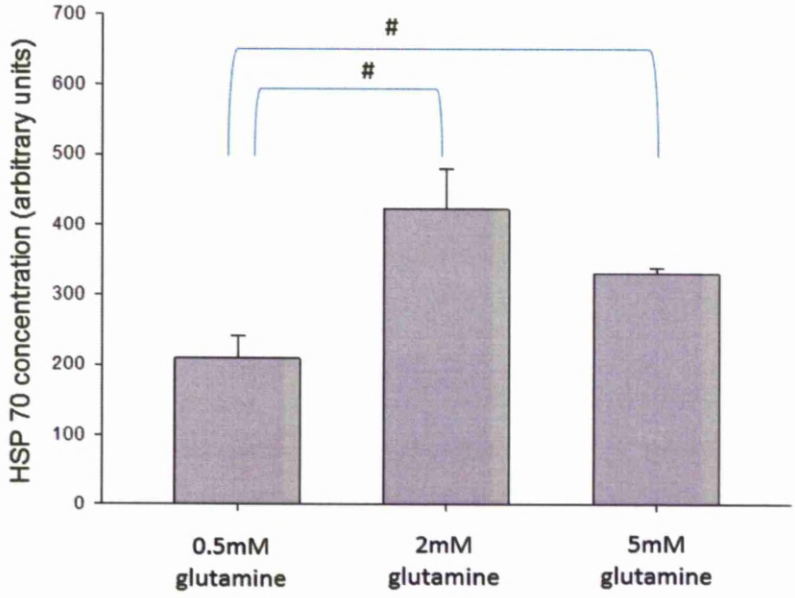


Figure 3.15 HSP 70 content of C2C12 myotubes after maintenance of glutamine depletion or subsequent repletion and following heat treatment. Intracellular HSP 70 content is expressed in arbitrary units. Data are presented as mean +/- SEM, n=5. $^{\#}p = < 0.05$.

Figure 3.16 shows HSC 70 content of C2C12 myotubes. Cells were seeded in differentiation media containing 2mM glutamine. At day 4 media was changed to media containing 0.5mM glutamine. At day 7 media was changed to media containing either 0.5mM, 2mM or 5mM glutamine. Myotubes were heat stressed at day 8 and harvested at day 9 (Figure 3.4). Data demonstrate that the HSC 70 content of heat treated cells in 5mM glutamine had a significantly higher content of HSC 70 compared with the HSC 70 content of heat treated cells in 0.5mM glutamine and 2mM glutamine. The HSC 70 content of heat treated cells in 2mM glutamine had a significantly higher content of HSC 70 compared with the HSC 70 content of heat treated cells in 0.5mM glutamine. These data suggest that the hyperthermia-induced HSC 70 content of cells was considerably reduced in cells which were maintained in glutamine deficient media and this was corrected with glutamine repletion.

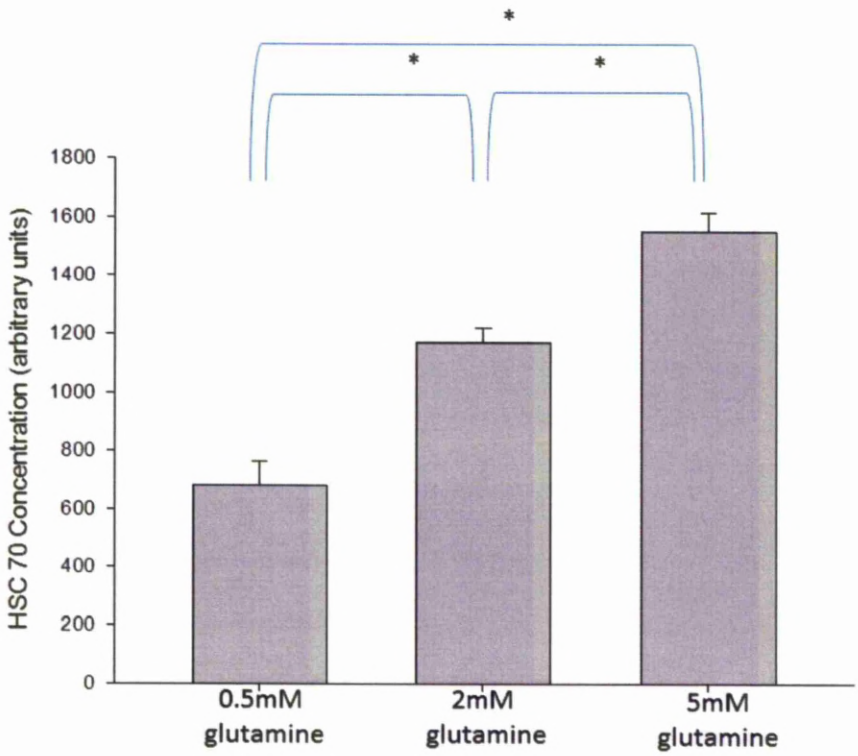


Figure 3.16 HSC 70 content of C2C12 myotubes after maintenance of glutamine depletion or subsequent repletion and following heat

treatment. Intracellular HSC 70 content is expressed in arbitrary units. Data are presented as mean +/- SEM, n=5. $^{*}p = < 0.05$.

Figure 3.17 shows the HSP 60 content of C2C12 myotubes in a similar manner to Figures 3.15-3.16. Intracellular HSP 60 content was not significantly different between the three conditions at day 9 of the experiment.

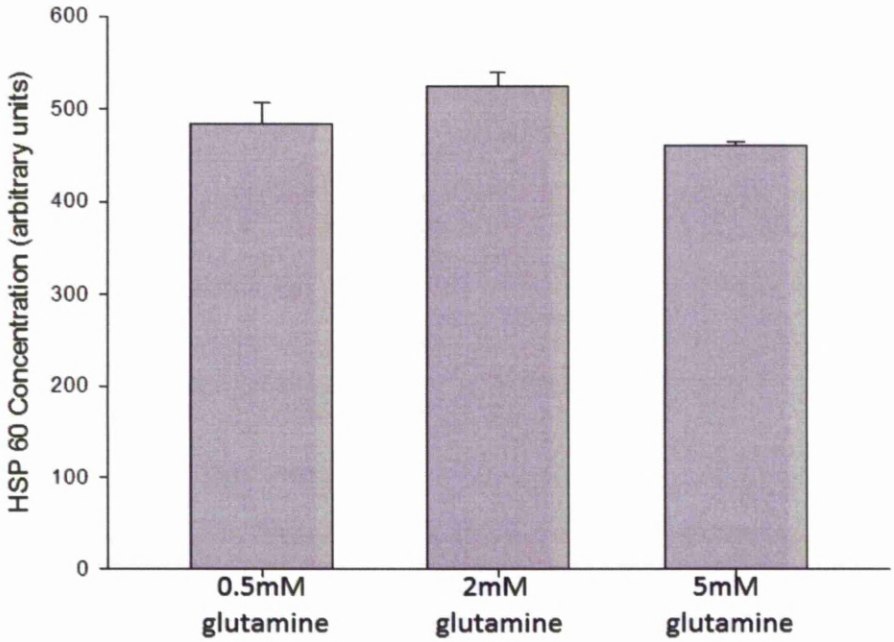


Figure 3.17 HSP 60 content of C2C12 myotubes after maintenance of glutamine depletion or subsequent repletion and following heat treatment. Intracellular HSP 60 content is expressed in arbitrary units. Data are presented as mean +/- SEM, n=4.

3.3.4. The effect of TNF-α treatment on HSP content and cytokine production of myotubes maintained in 2mM glutamine.

Figure 3.18 shows representative images of myotubes grown and maintained in media containing 2mM glutamine at 24 hours following treatment with different concentrations of TNF-α. After seeding in differentiation media glutamine concentration was kept constant at 2mM glutamine. All other components in the medium were maintained at same concentrations during the experiment (Section 2.1). Media were changed every 48hours. At day 8 media was changed to media containing either 10ng/ml, 25ng/ml, 50ng/ml or 100ng/ml (Figure 3.5). Myotube appearance was not overtly altered at the different TNF-α concentrations.

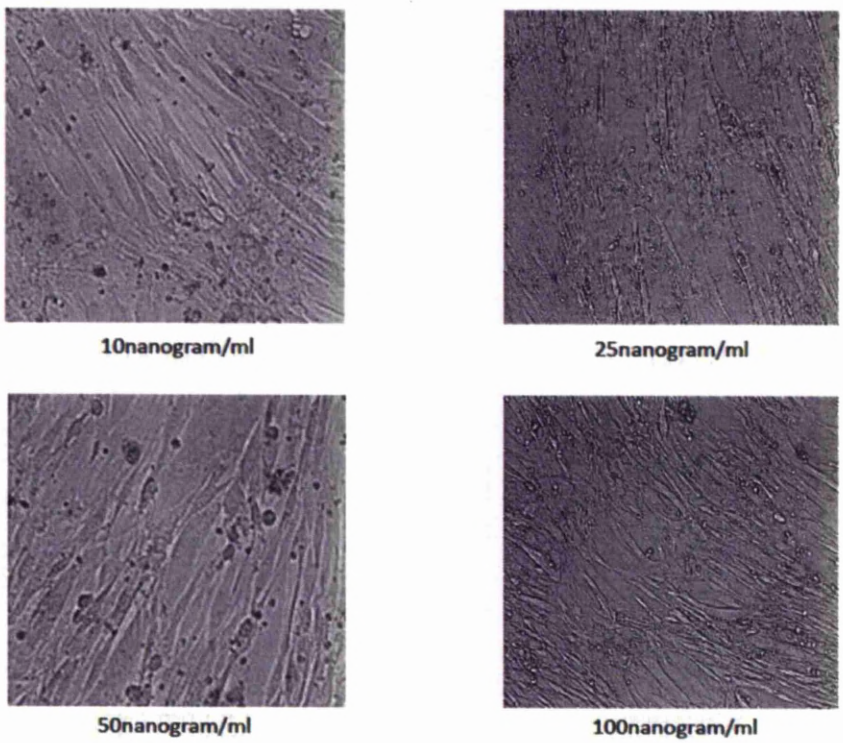


Figure 3.18 The effect treatment of C2C12 myotubes with different TNF-α concentrations on myotube structure (10x). Glutamine concentration was kept constant at 2mM glutamine throughout the experiment.

3.3.5. The effect of TNF-α treatment on HSP content of myotubes maintained in 2mM glutamine.

Initial studies established that treatment of myotubes with 10ng/ml TNF-α resulted in a significant increase in HSP content of cells compared with the HSP content of untreated cells (Figure 3.19). This treatment regime was then used for all further studies and comparisons presented reflect the HSP content of cells maintained in a glutamine deficient media or in a replete media and subjected to treatment with TNF-α compared with cells maintained in 'normal' 2mM glutamine and treated with TNF-α.

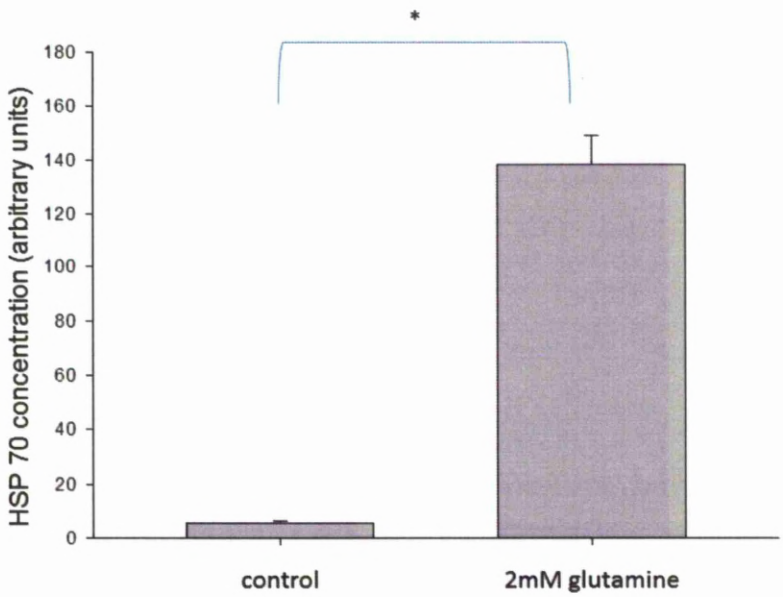


Figure 3.19 HSP 70 content of C2C12 myotubes after glutamine depletion (0.5mM) and subsequent repletion (2mM) and treatment with TNF-α compared with no TNF-α treatment. Intracellular HSP 70 content is expressed in arbitrary units. Data are presented as mean +/- SEM, n=5. $^{*}p = < 0.05$.

Figures 3.20-3.22 show the HSP 70, HSC 70 and HSP 60 content of C2C12 myotubes at 24hours following treatment with different TNF-α concentrations. After seeding in differentiation media glutamine concentration was kept constant at 2mM glutamine. Media were changed every 48hours. At day 8 media was changed to media containing either 10nanog/ml TNF-α, 25ng/ml TNF-α, 50ng/ml TNF-α or 100ng/ml TNF-α (see Figure 3.5). All other components in the medium were maintained at same concentrations during the experiment (Section 2.1.1). Intracellular HSP 70, HSC 70 and HSP 60 content was not significantly different between the four conditions suggesting a similar induction of HSPs following treatment with all concentrations of TNF-α.

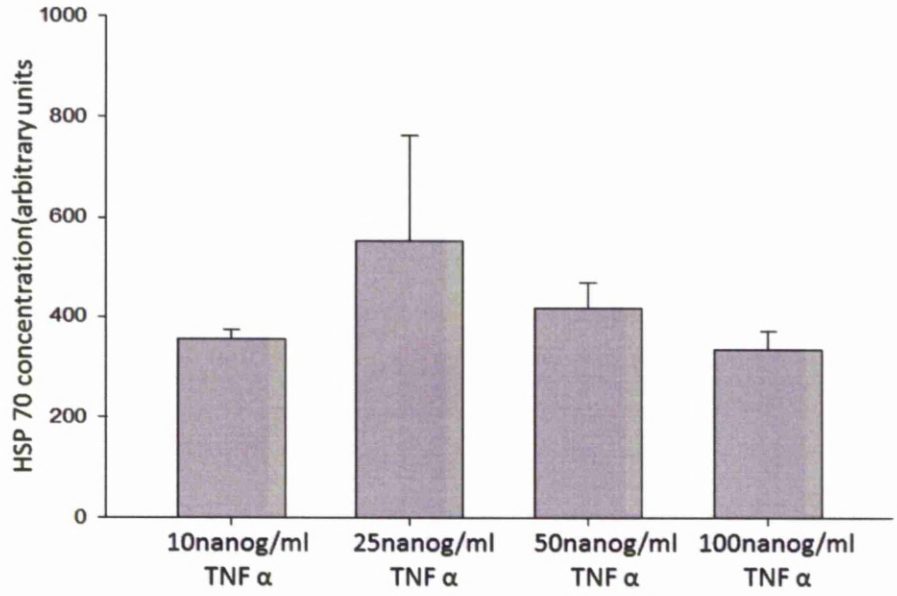


Figure 3.20 HSP 70 content of C2C12 myotubes after treatment with different concentrations of TNF-α. Glutamine concentration was kept constant at 2mM glutamine. Intracellular HSP 70 content is expressed in arbitrary units. Data presented as mean +/- SEM, n=5.

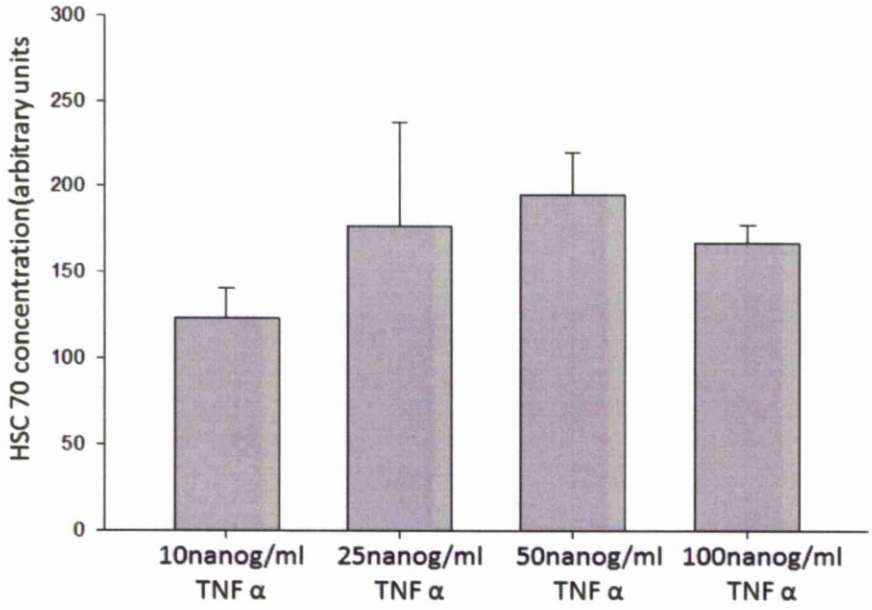


Figure 3.21 HSC 70 content of C2C12 myotubes after treatment with different concentrations of TNF-α. Glutamine concentration was kept constant at 2mM glutamine. Intracellular HSC 70 content is expressed in arbitrary units. Data presented as mean +/- SEM, n=5.

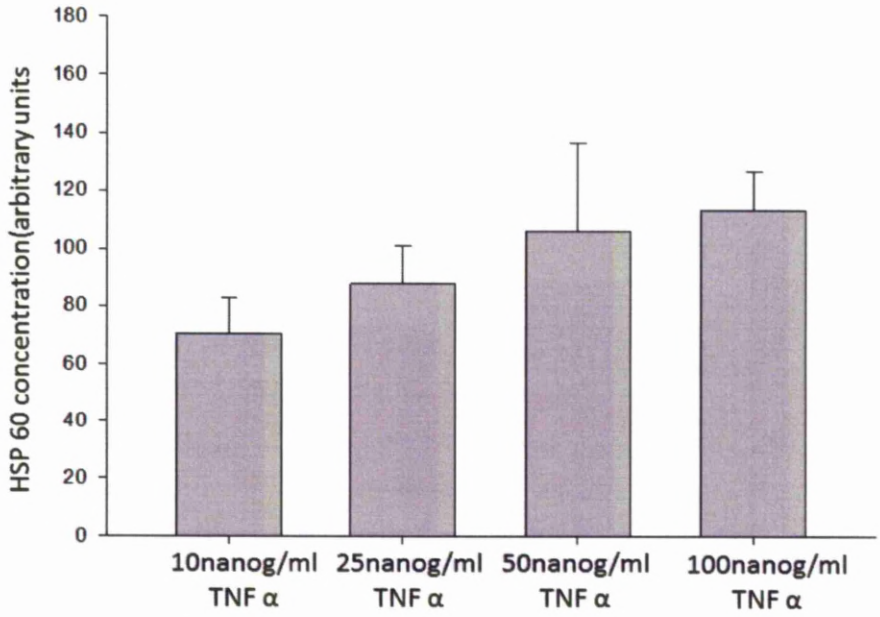


Figure 3.22 HSP 60 content of C2C12 myotubes after treatment with different concentrations of TNF-α. Glutamine concentration was kept constant at 2mM glutamine. Intracellular HSP 60 content is expressed in arbitrary units. Data presented as mean +/- SEM, n=5.

3.3.6. The effect of TNF-α treatment on cytokine release by myotubes maintained in 2mM glutamine

Figures 3.23 - 3.25 and Table 3.1 show the concentrations of TNF-α, IL 1beta and IL 6 in media from C2C12 mouse myotubes at 24 hours following treatment with different concentrations of TNF-α. Glutamine concentration was kept constant at 2mM glutamine. Data demonstrated that the TNF-α concentration in media following treatment with 25ng/ml TNF-α was higher than treatment with 50nanog/ml TNF-α or 100nanog/ml TNF-α respectively, p = 0.01. No cytokines were detectable in untreated cells and no difference

was detected in the IL 1beta and IL 6 concentrations in media following treatment with different concentrations of TNF-α.

	10ng/ml TNF α	25g/ml TNF α	50ng/ml TNF α	100ng/ml TNF α
TNF α	1392 +/- 43 pg/ml	1441 +/- 31 pg/ml	1194 +/- 45 pg/ml	1053 +/- 12 pg/ml
IL 1beta	8.07 +/- 0.66 pg/ml	7.63 +/- 0.77 pg/ml	7.50 +/- 3.64 pg/ml	8.71 +/- 0.61 pg/ml
IL 6	79 +/- 12 pg/ml	87 +/- 41 pg/ml	124+/- 54 pg/ml	80 +/- 26 pg/ml

Table 3.1 Concentrations of TNF-α, IL 1beta and IL 6 at 24 hours following treatment with different concentrations of TNF-α. Cytokine concentrations presented as mean +/- SEM.

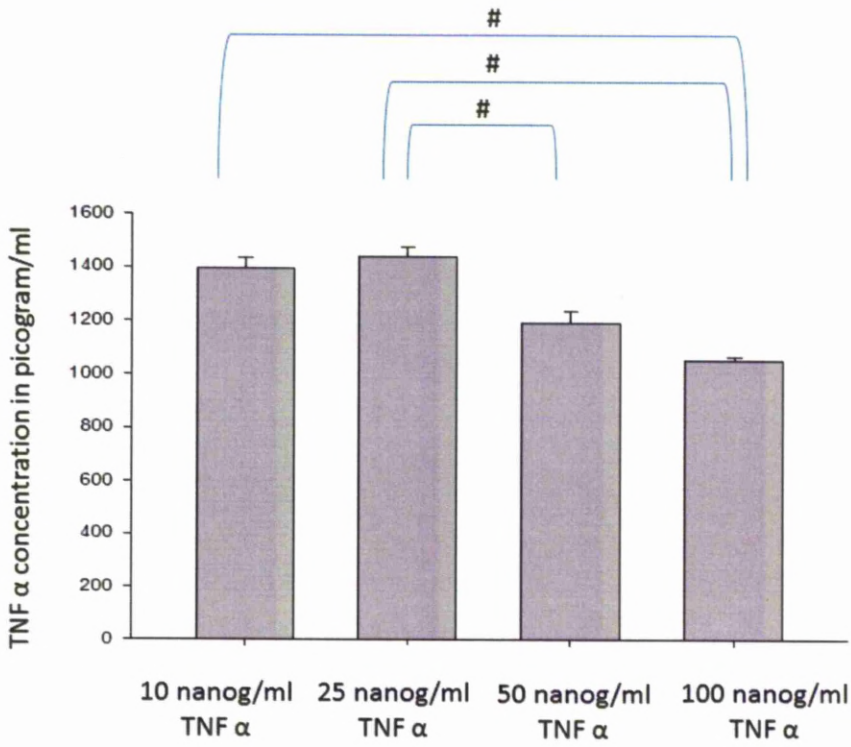


Figure 3.23 Media TNF-α concentration at 24 hours following treatment with different concentrations of TNF-α. Glutamine concentration was kept constant at 2mM glutamine. TNF-α concentration presented as mean +/- SEM. Data presented as mean +/- SEM, n=3. $^{\#}p = < 0.05$.

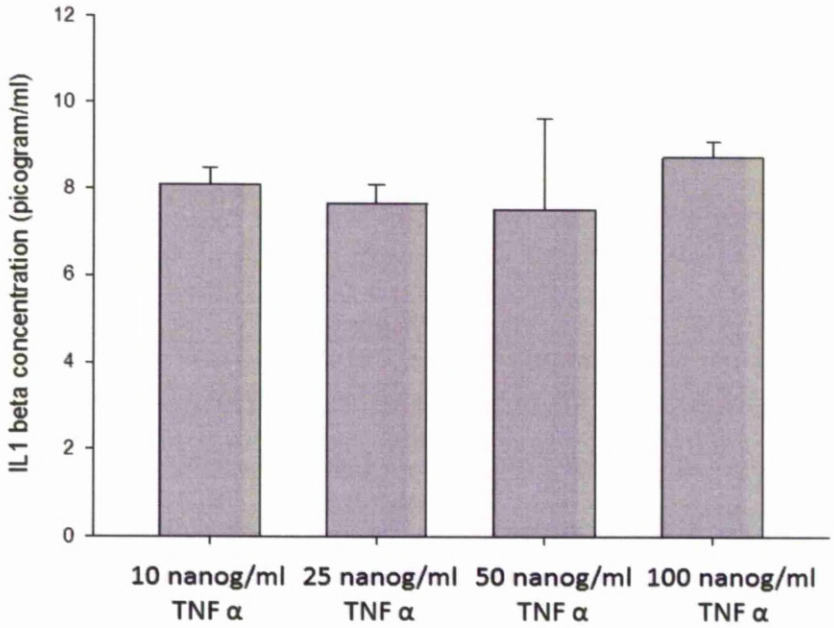


Figure 3.24 Media IL1β concentration at 24 hours following treatment with different concentrations of TNF-α. Glutamine concentration was kept constant at 2mM glutamine. Data presented as mean +/- SEM, n=3.

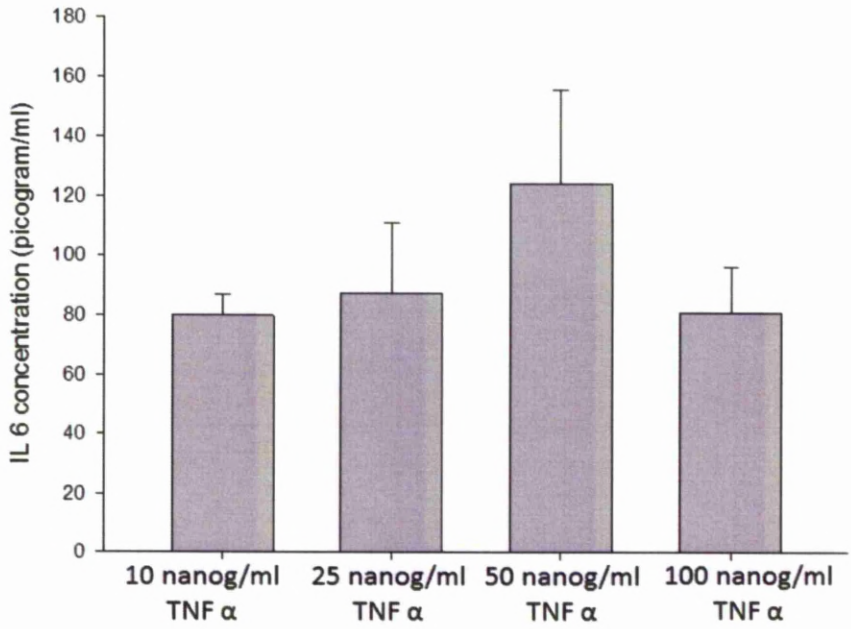


Figure 3.25 Media IL 6 concentration at 24 hours following treatment with different concentrations of TNF-α. Glutamine concentration was kept constant at 2mM glutamine. Data presented as mean +/- SEM, n=3.

3.3.7. The effect of different concentrations of extracellular glutamine on intracellular HSP concentration of myotubes following treatment with TNF-α.

No significant difference in intracellular HSP concentration was seen following treatment with the different concentrations of TNF-α (Section 3.3.3). Although there were significant differences observed in the cytokine release into medium after treatment of cells with 10ng/ml and 25ng/ml, compared with 50 and 100ng/ml TNF-α, 10nanog/ml TNF-α was felt to be the most appropriate concentration in the following experiment as this level is physiologically relevant.

Figure 3.26 shows representative images of the effect of different concentrations of glutamine on myoblast structure at 24hours following treatment with 10ng/ml TNF-α.

Cells were seeded in differentiation media containing 2mM glutamine. At day 4, media was changed to media containing 0.5mM glutamine. At day 7 media was changed to media containing 0.5mM, 2mM or 5mM glutamine to mimic maintained depletion or repletion. Myotubes were then treated with 10ng/ml TNF-α and harvested 24 hours later (Figure 3.6). Myotube appearance was not overtly altered between the different glutamine concentrations.

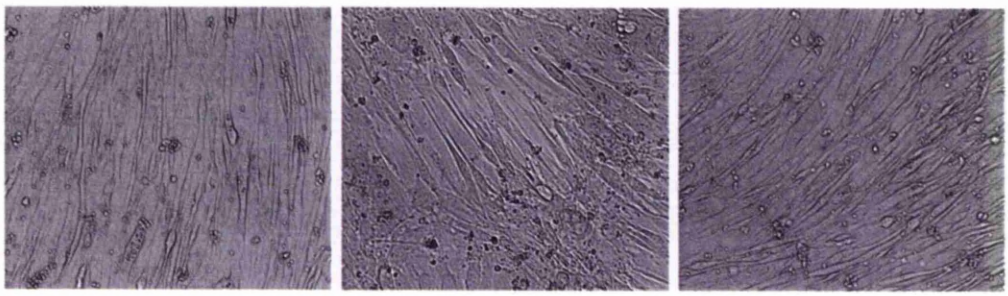


Figure 3.26 The effect of different concentrations of extracellular glutamine on myotubes after maintenance of glutamine depletion or subsequent repletion and following treatment with TNF-α (10x).

3.3.8. The effect of different concentrations of extracellular glutamine on intracellular HSP concentration of myotube following treatment with TNF-α.

Figure 3.27 shows the HSP 70 content of C2C12 myotubes. Cells were seeded in differentiation media containing 2mM glutamine. At day 4 media was changed to media containing 0.5mM glutamine. At day 7 media was changed to media containing 0.5mM, 2mM or 5mM glutamine. Myotubes were stressed with 10ng/ml TNF-α at day 8 and harvested at day 9 (Figure 3.6). Intracellular HSP 70 was not significantly different between the three TNF-α treated groups. Data presented as mean +/- SEM, n=4. $^{*}p = < 0.05$.

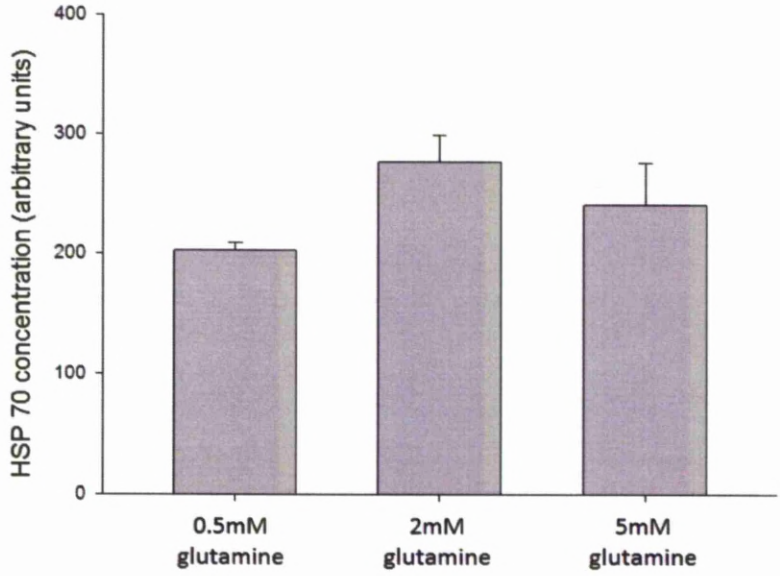


Figure 3.27 HSP 70 content of C2C12 myotubes after maintenance of glutamine depletion or subsequent repletion and following treatment with TNF-α. Intracellular HSP 70 content is expressed in arbitrary units. Data presented as mean +/- SEM, n=4.

Figure 3.28 shows HSC 70 content of C2C12 myotubes. Data demonstrate that intracellular HSC 70 content was not significantly different between the three TNF-α treated groups.

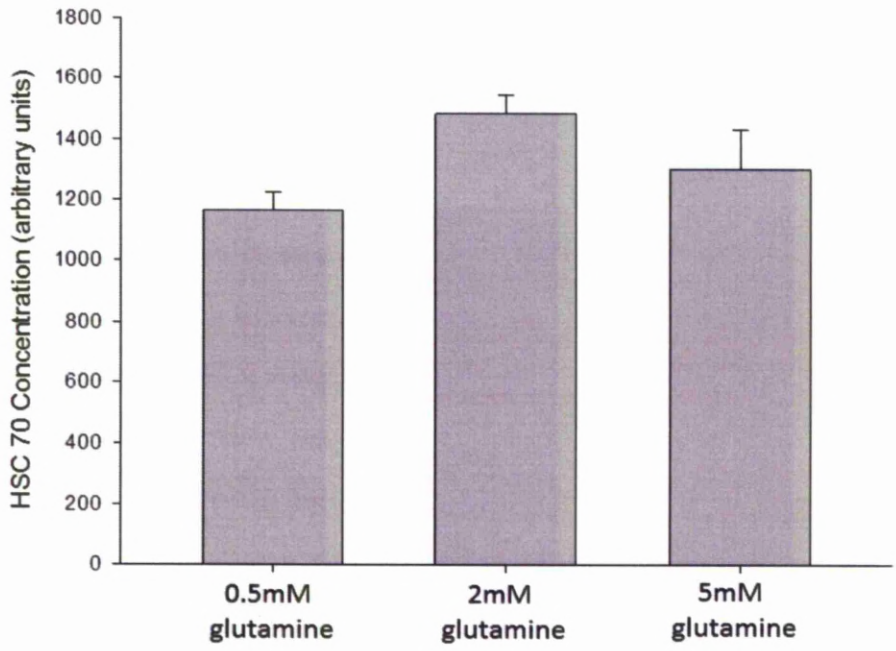


Figure 3.28 HSC 70 content of C2C12 myotubes after maintenance of glutamine depletion or subsequent repletion and following treatment with TNF-α. Intracellular HSC 70 content is expressed in arbitrary units. Data presented as mean +/- SEM, n=4.

Figure 3.29 shows the HSP 60 content of C2C12 myotubes. Data demonstrate that intracellular HSP 60 content was not significantly different between the three TNF-α treated groups.

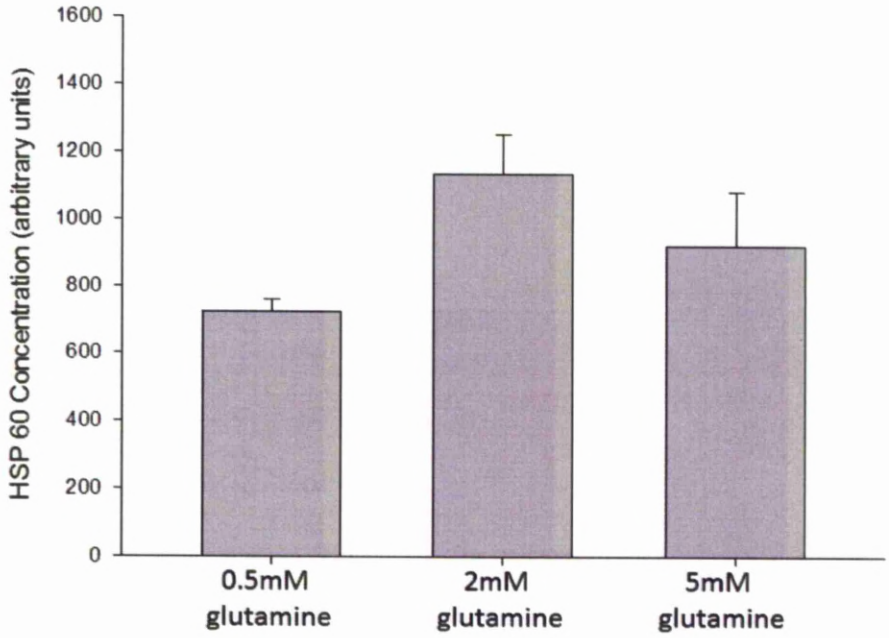


Figure 3.29 HSP 60 content of C2C12 myotubes after maintenance of glutamine depletion or subsequent repletion and following treatment with TNF-α. Intracellular HSP 60 content is expressed in arbitrary units. Data presented as mean +/- SEM, n=4.

3.3.9. The effect of different concentrations of extracellular glutamine on cytokine production by myotubes following treatment with TNF-α.

Figures 3.30 – 3.32 show the concentrations of TNF-α, IL β and IL 6 in the media of C2C12 mouse myotubes following treatment with TNF-α. Cells were seeded in differentiation media containing 2mM glutamine. At day 4 media was changed to media containing 0.5mM glutamine. At day 7 media was changed to media containing 0.5mM, 2mM or 5mM glutamine. Myotubes

were treated with 10ng/ml TNF-α at day 8 and harvested 24 hours later (Figure 3.6).

Data demonstrate that the TNF-α concentration of media from myotubes treated with 10ng/ml TNF-α and repleted with 2mM glutamine had a significantly higher TNF-α release compared with media of myotubes maintained in 0.5mM or repleted with 5mM glutamine. Data demonstrate IL 1beta and IL 6 concentration of media from myotubes treated with TNF-α was not significantly different between the three TNF-α treated groups.

10nanog/ml TNF α	0.5mM glutamine	2mM glutamine	5mM glutamine
TNF α	271 +/- 37	584 +/- 56	286 +/- 39
IL 1beta	9.98 +/- 0.405	8.69 +/- 0.40	9.22 +/- 1.297
IL 6	8.17 +/- 1.69	15.06 +/- 2.92	12.63 +/- 1.40

Table 3.2 Media concentrations of TNF-α, IL 1beta and IL 6 at 24 hours following treatment with TNF-α. Concentrations presented as mean (pg/ml) +/- SEM.

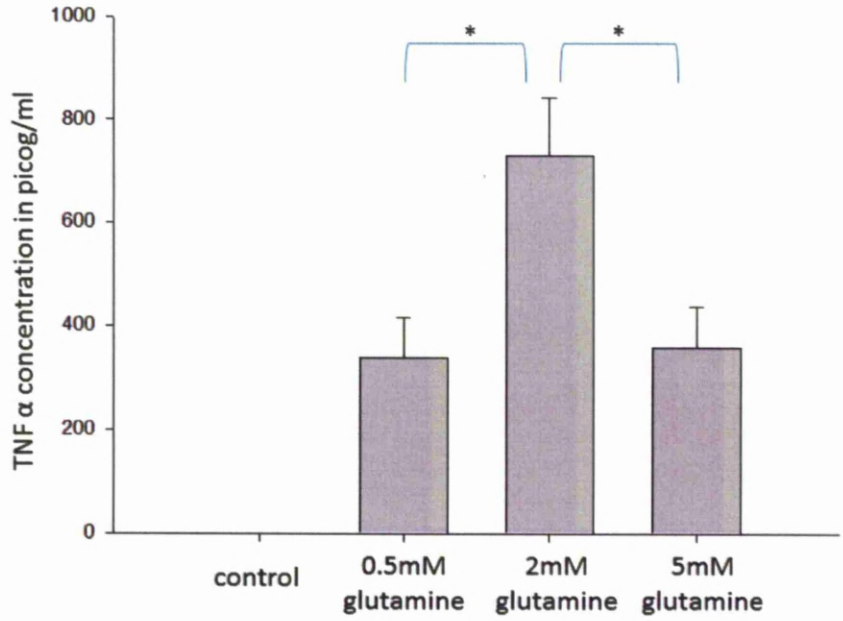


Figure 3.30 Media concentration of TNF-α at 24 hours following treatment of C2C12 cells maintained depleted or replete with glutamine. Data presented as mean +/-SEM, n=4. $^{*}p = < 0.05$.

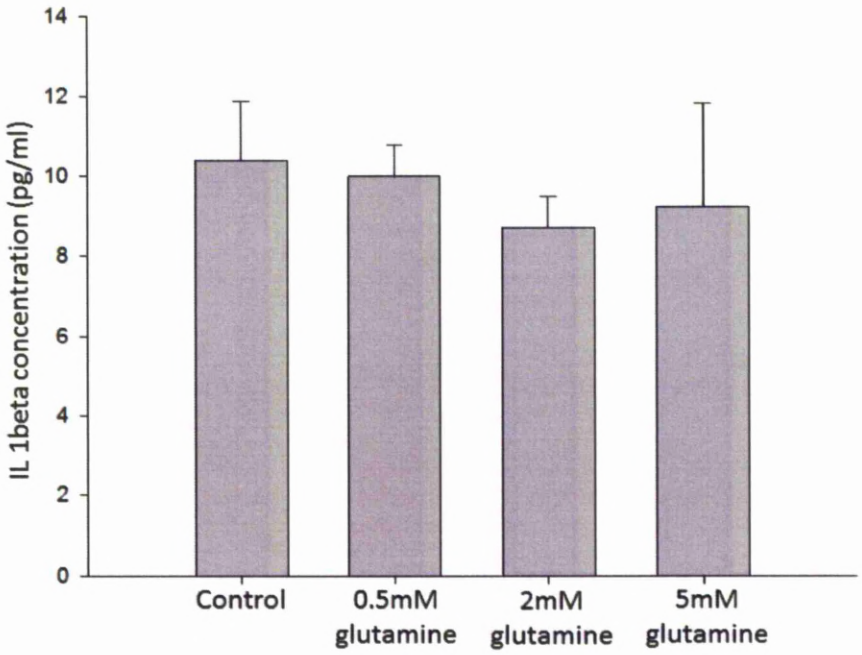


Figure 3.31 Media concentration of IL 1β at 24 hours following treatment of C2C12 cells maintained depleted or replete with glutamine. Data presented as mean +/-SEM, n=4.

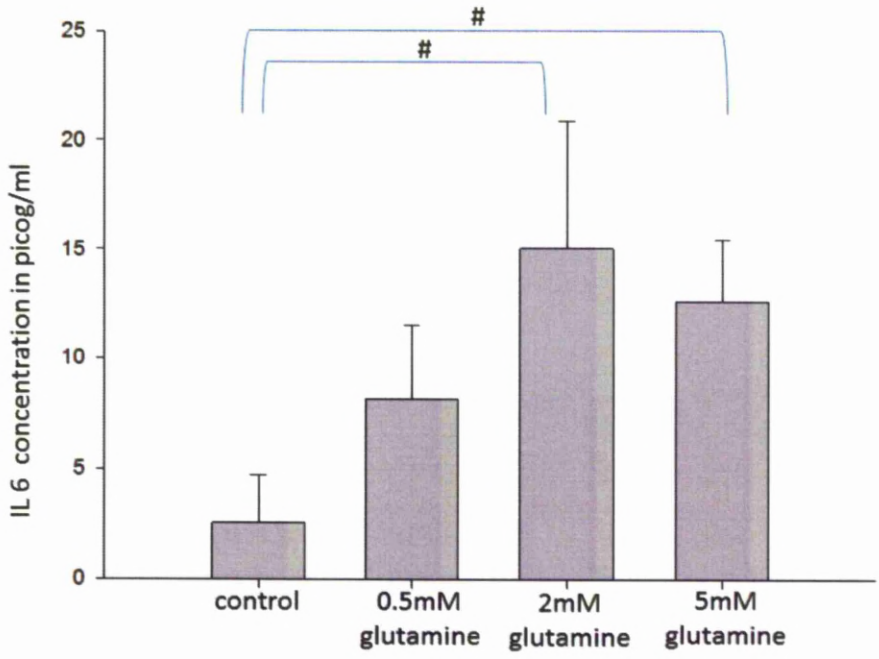


Figure 3.32 Media concentration of IL 6 at 24 hours following treatment of C2C12 cells maintained depleted or replete with glutamine. Data presented as mean +/-SEM, n=4. $^{\#}p = < 0.05$.

3.4 Discussion

Myoblasts undergo significant remodelling to form mature myotubes with the increased expression of a number of muscle specific proteins and so relatively high HSP content of myoblasts during this differentiation and maturation phase has previously been observed (Maglara et al. 2003). Glutamine has been implicated to play a role in modulating the intracellular levels of HSPs although the effects of modified glutamine concentrations on muscle cell growth and maturation has not been examined in detail. This study showed that low extracellular glutamine concentrations inhibited maturation and, not unexpectedly, gross extracellular deficiency resulted in an inability to produce HSPs and cell death. In contrast, marginal extracellular glutamine deficiency,

i.e. extracellular glutamine concentrations of 0.5mM and 1mM glutamine, so glutamine concentrations a quarter and half of normal glutamine concentrations, appeared to induce a higher level of HSPs in muscle cells, possibly reflecting that the cells were stressed at levels of relative deficiency. Little effect of modified levels of glutamine on HSP60 content was observed. HSP60 is primarily localised to the mitochondria and so data suggest that modification of glutamine has little effect on mitochondrial numbers or the mitochondria specific stress response.

This study also addressed the effect of either (i) 48hours of relatively high (2.5 x normal) glutamine concentration or (ii) continued depletion of glutamine, after a period of relative deficiency in an unstressed cell resulted in modified HSP content compared with normal extracellular glutamine concentration and the effect of this on the ability to respond to additional stress of either hyperthermia using a standard protocol or to treatment with TNF-α to mimic sepsis. To facilitate this, C2C12 myoblasts were grown in medium containing 2mM glutamine until maturity (day 4 post addition of differentiation media) and then the medium was changed from 2mM to 0.5mM to resemble a modest glutamine deficiency (Biolo et al. 2005). This concentration of 0.5mM glutamine, 25% of normal, was used to facilitate a low glutamine concentration and therefore resemble a relative deficiency as seen in a clinical setting. A similar concentration of 25% of the physiological glutamine concentration has been used by other researchers in a human leucocyte model (Oehler et al. 2002). Interestingly, little effect of this acute depletion and repletion was seen on myotube HSP content.

Numerous environmental and pathological stressors including heat and TNF-α are known to increase the intracellular content of HSPs in a range of cell types (Morimoto et al. 1992) where the HSPs are accepted to play a major important role in cellular protection and repair. Preconditioning i.e. facilitating an increased intracellular HSP concentration prior to damaging stress has been shown to increase cell protection and in turn cell survival (Maglara et al. 2003). This survival benefit has been shown in several animal sepsis models (Villar et al. 1994; Chu et al. 1997; Weiss et al. 2002; Wang et al. 2007). Glutamine has been implicated to augment cell survival against a

variety of stressful stimuli and this beneficial effect has been proposed to involve increased intracellular HSP 70 concentrations (Wischmeyer et al. 1997; Musch et al. 1998). Indeed, intravenous glutamine administration correlates with an increased content of HSP 70 in several organs of the rat. Importantly a dose dependant response of HSP 70 to glutamine dose was observed (Wischmeyer et al. 2001). This observation supported the view that glutamine has the remarkable ability to work as pharmacological agent facilitating higher intracellular HSP contents with higher administrated glutamine doses (Wischmeyer. 2006b). Other authors have suggested that the observed deficiency of glutamine during critical illness as modelled by a cell culture model and an animal sepsis model is responsible for the attenuated HSP 70 response (Eliasen et al. 2006a; Oehler et al. 2002; Wang et al. 2007)

The experiments described in this chapter have addressed the above questions in a muscle cell culture model, namely the question whether extracellular glutamine concentrations correlate with intracellular HSP concentrations and therefore whether glutamine has pharmacological properties or whether extracellular glutamine deficiency attenuated the intracellular HSP concentration. Thus, substitution of normal glutamine (2mM) in cell culture at a stage of myoblast maturity compared with a deficiency (0.5mM) did not influence HSP expression in the unstressed cell.

Data demonstrate that cells maintained in normal (2mM) glutamine were able to mount a stress response by significantly increasing the intracellular HSP following treatment with either heat or TNF-α and this effect was blunted by between 25 and 40% in cells which were maintained in a relatively glutamine deficient media at 0.5mM glutamine. In contrast, no appreciable difference between the intracellular HSP content was seen between cells maintained at 2mM extracellular glutamine compared with 5mM extracellular glutamine with the exception of HSP 70 whereby intracellular HSC 70 content after heat stress was significantly greater in myotubes in medium containing 5mM glutamine compared with medium containing 2mM glutamine. These results support the importance of maintaining normal glutamine concentrations during stress. Thus, no major difference could be observed between cells maintained at 2mM glutamine compared with 5mM glutamine, suggesting no

added benefit of maintaining glutamine levels at more than twice normal level.

This study demonstrated a significant increase in TNF-α production and release by muscle cells replete with 2mM glutamine compared with cells which were either maintained at 0.5mM glutamine or replete with 5mM glutamine. It may be counter-intuitive to measure TNF-α release by cells treated with TNF-α, but evidence suggests that muscle cells respond to increases in extracellular TNF-α such as during sepsis by the increased production of a number of pro- and anti-inflammatory cytokines including TNF-α. Pilot data from our laboratory (personal communication, data not shown) have demonstrated that the TNF-α added to conditioned media is degraded within a short time course and the TNF-α measured in the media in this experiment reflects chronic release by C2C12 cells. It is not clear whether the observed decrease in TNF-α release from cells maintained at 0.5mM glutamine or repleted with 5mM glutamine is due to a direct effect of glutamine on TNF-α expression as suggested by others (Wischmeyer et al. 2003). However, it is known that an attenuated as well as an overwhelming TNF-α response to stress can also be detrimental. Therefore a significant rise after exposure of cells replete with 2mM glutamine and treated with 10ng/ml TNF-α might represent an adequate response compared to the response seen in the 0.5mM glutamine and the 5mM glutamine groups.

Thus, in summary, correction of an extracellular glutamine deficiency rather than increases to twice normal extracellular glutamine concentrations appear to be the major influence on muscle maturation and ability to raise intracellular HSP content following heat and TNF-α stress.

CHAPTER 4

CLINICAL STUDY

4.1 Introduction

4.1.1 Critical care medicine

Patients admitted to intensive care units suffer from a diverse range of conditions ranging from those admitted electively following planned major surgery to those admitted as emergencies following some surgical catastrophe, major trauma, sepsis or respiratory failure. The variation in age range and prior health status is extreme and nowadays Critical Care Units are admitting increasingly more elderly, frail or malnourished patients with a median age of 63years (Harrison et al. 2004).

Importantly, critical illness caries a significant mortality risk. Data from 129,647 admissions to UK critical care units showed a critical care unit mortality of 20.3% and a hospital mortality of 30.8%.

4.1.2 Glutamine and critical illness

In critical illness, glutamine has been postulated to become conditionally essential as the increased demand cannot be met by synthesis (see Section 1.3.1; Griffiths. 2004). Indeed reduced glutamine concentrations have been observed in serum and tissues during critical illness (Jackson et al. 1999) and low plasma glutamine concentrations have been associated with increased mortality in critical illness (Oudermans van Straaten et al. 2001; Rodas P et al, 2012).

Intravenous glutamine administration has been suggested to be beneficial in severe critical illness and has therefore been recommended as part of a par-enteral nutrition regime (Heyland et al. 2009. Due to the publication of further parenteral glutamine trials, this advice has recently been challenged (see chapter 1.3.1; Kent and Bongers. 2011).

4.1.3 Effects of glutamine administration on the stress response

The various effects of glutamine administration have been discussed in detail in Section 1.3. It is however important to outline the proposed glutamine effects that led to the design of the pilot clinical trial described in this chapter (Section 2.1.2).

Glutamine appears to regulate protein turnover in myotubes cultures, increasing the half-life of long-lived proteins. This has been proposed to be related to the glutamine-induced increase in highly inducible HSP70 (Zhou and Thompson. 1997). Further, glutamine appears to be a potent enhancer of the HSP response (Nissim et al. 1993; Wischmeyer et al. 1997; Musch et al. 1998). Intravenous glutamine infusions, over a range of doses (0.15-0.75 g/kg), are able to enhance HSP concentrations in multiple organs of the rat in a dose dependant manner (Wischmeyer et al. 2001). This induction occurs as early as 1 hour post-administration and persists for up to 72 hours post-administration. Moreover, intravenous glutamine infusion prior to a septic insult was associated with protection against endotoxin-induced septic shock in the rat and could markedly decrease end-organ injury and overall mortality (Wischmeyer et al. 2001). It could further be shown that glutamine given post-septic insult enhanced HSP 70 and 25 expression, protected against acute lung injury and reduced end-organ injury and overall mortality (Singleton et al. 2005b). Importantly, the survival benefit from glutamine was abrogated if a HSP inhibitor was administered. Indeed it has been suggested that glutamine deficiency renders the cells incapable of an adequate HSP response (Oehler et al. 2002).

Consequently it has been suggested that glutamine substitution influences the HSP response in humans and indeed the observed survival benefit of glutamine added to parenteral nutrition to meet a developing deficiency in the critically ill might reflect this. Moreover it has been demonstrated that glutamine substitution correlates with increased HSP 70 response in the critically ill and suggests an improved clinical outcome (Ziegler et al. 2005).

The ability of cells to induce HSPs following stress is reduced in aged humans and animals (Heydari et al. 2000). Tissues from aged animals and blood cells from elderly humans both show a reduced production of stress proteins

following stress (Soti and Csermely. 2003; McConnell et al. 2011) Further, reduced glutamine concentration is an independent predictor of mortality in critical illness (Rodas et al. 2012; Oudermans Van Straaten et al. 2001). However, the low concentration glutamine group was significantly older (Oudermans van Straaten et al. 2001). A low HSP concentration in severe trauma is correlated with increased mortality. But again, the high mortality group was significantly older (Pittet et al. 2002). This lack of adaptation in HSP content in the aged animals may be related to a more general failure of adaptation to stress.

4.1.4. Effect of HSPs on pro-inflammatory cytokine production

Increased HSP expression has been shown to attenuate plasma concentrations of the pro-inflammatory cytokines IL-1β and TNF-α, both *in vitro* and *in vivo* models and this appears to correlate with improved survival from septic insults (Chu et al. 1997) and recent (unpublished) data from our laboratory provides evidence for muscle as a major source of pro- and anti-inflammatory cytokines. In addition to their local intracellular role, HSPs participate in cytokine signalling, cytokine gene expression and enhance antigen presentation to T lymphocytes (Pockley. 2003; De Maio. 2011; for detailed discussion Section 1.4.5).

4.1.5 Early administration of glutamine

Intravenous glutamine is administered as part of parenteral nutrition (intravenous nutrition), which is not usually commenced until several days after admission to critical care. This is in line with current recommendation regarding nutrition during critical illness (Heyland et al. 2009) as parenteral nutrition is not recommended as first line treatment unless gastrointestinal failure is present rendering the patient incapable to absorb nutrition via the gastrointestinal route. The diagnosis of gastrointestinal failure is currently established after at least one of the following gastrointestinal problems: food intolerance, gastrointestinal haemorrhage or ileus (Reintam et al. 2006).

However, as outlined above a HSP response after glutamine administration could be observed as early as 1hour after administration in a rat sepsis model (Wischmeyer et al. 2001). Importantly, an increased tissue HSP content could be observed even when glutamine was administered after the septic insult (Singleton et al. 2005b) suggesting that glutamine administration may be beneficial as early as possible after admission to critical care.

4.1.6 Establishment of the optimal glutamine dose

In health, glutamine is a nonessential amino-acid with a daily whole body turnover of about 100g per day. It has however been widely accepted that glutamine is a crucial component of parenteral nutrition in the critically ill patient (Heyland et al. 2009; Section 1.3.6). It has been suggested that the supplementation dose should be greater than 0.2 g per kg bodyweight per day and others suggest at least 0.4g per kg per day (Glutamine supplementation. 2009). However, even higher glutamine doses of up to 0.86g per kg bodyweight/ day have been proposed to be safe (Tjaeder et al. 2004).
This study was designed to answer the question whether one large glutamine dose early in critical illness can affect the serum and muscle tissue HSP content. Peak HSP tissue contents have been observed between 24-48 hours after administration of one large single dose of glutamine (Wischmeyer et al. 2001). The ideal time point for tissue and blood sampling was therefore chosen as 48hours after glutamine administration to identify the maximum effect of the intervention. The second time point after glutamine administration (96hours) was used as a further control assuming that one large dose of glutamine has no considerable effect beyond 72hours post glutamine administration.

4.1.7. Aim of study

This clinical study investigated whether administration of one large dose of intravenous glutamine (0.5g/kg bodyweight) as early as possible in critical illness resulted in modified HSP content in blood and in muscle tissue at

48hours and 96hours after randomisation and whether this was associated with improved survival.

4.2. Methods

4.2.1 Trial design

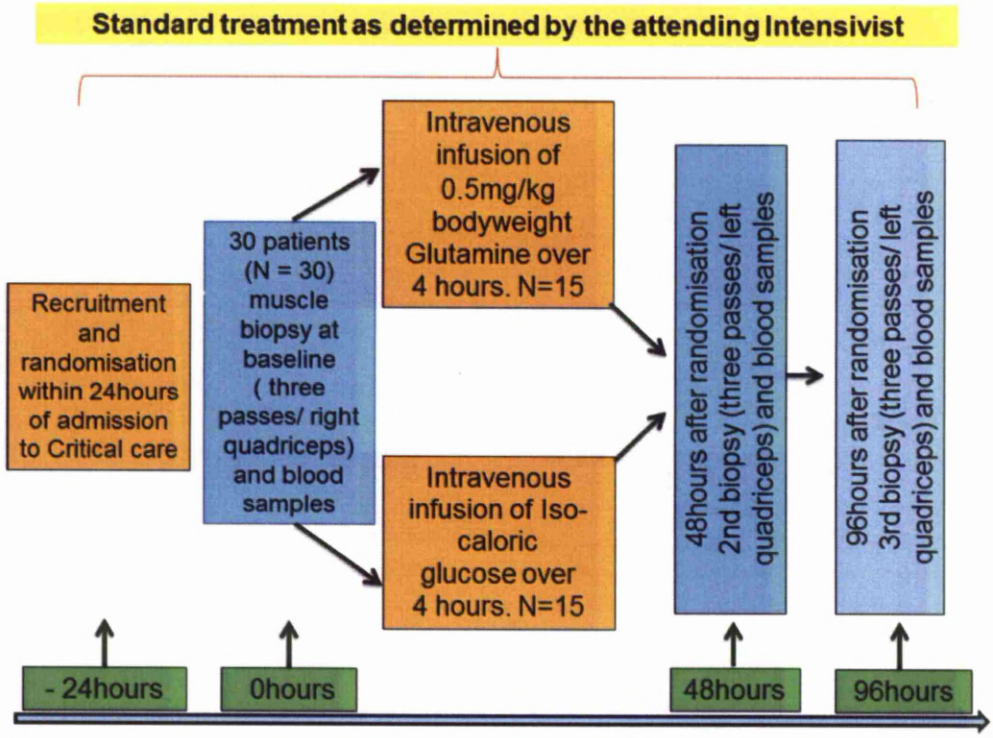


Figure 4.1: Schematic illustration of clinical trial

4.2.2 Randomisation

Randomisation of patients was controlled for age as approximately half of severely ill intensive care patients are over 65 years with upwards of 25% over 75 years of age (Harrison et al. 2004).

4.2.3. Recruitment

Figure 4.2 illustrates the screening and recruitment of patients for the clinical trial. Seventy two patients were screened for eligibility. Nine patients were excluded as they were likely to die within 48hours, 7 patients had elevated liver enzymes at the time of screening, 2 patients were suffering from concurrent malignancy, 2 patients were prioritised to a concurrent study and 2 patients were on total parenteral nutrition at the time of screening. It was impossible to contact the relatives of 13 patients within 24hours of admission to gain assent. Assent was not granted in 22 patients. 15 patients were entered into the study (Figure 4.2).

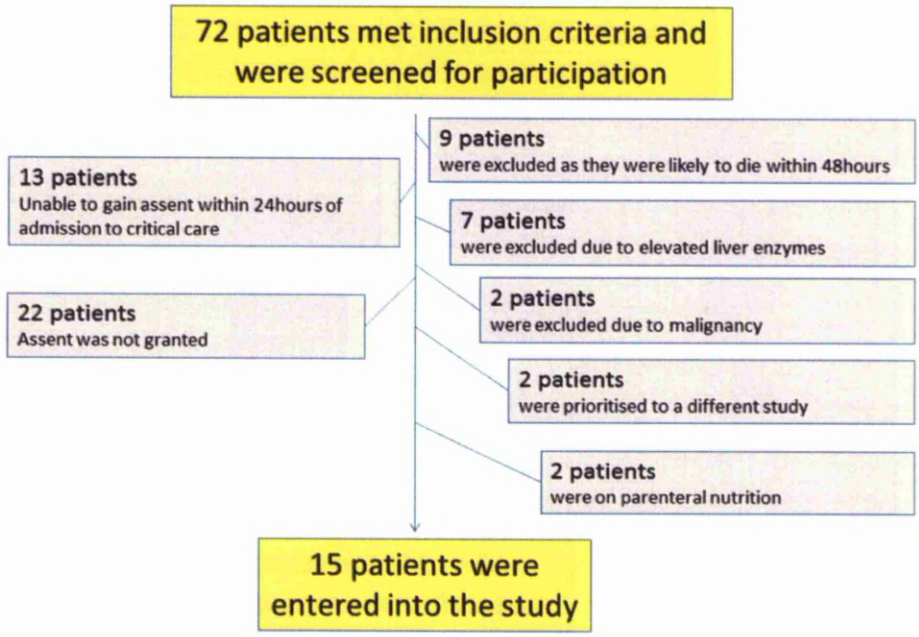


Figure 4.2: Illustration of screening and recruitment of clinical trial

Figure 4.3 illustrates the randomisation of patients for the clinical trial and the clinical outcome during their critical care stay. Seven patients were randomised to receive the isocaloric glucose infusion (placebo), eight patients were randomised to receive the intravenous glutamine infusion. Two patients

in the placebo group died within 48hours of randomisation, samples of one patient could not be used due to infection risk; Three patients in the glutamine group were moved to the ward before the end of sampling, one patient died within 48hours of randomisation leaving four patients per treatment group with a complet set of samples (Figure 4.3).

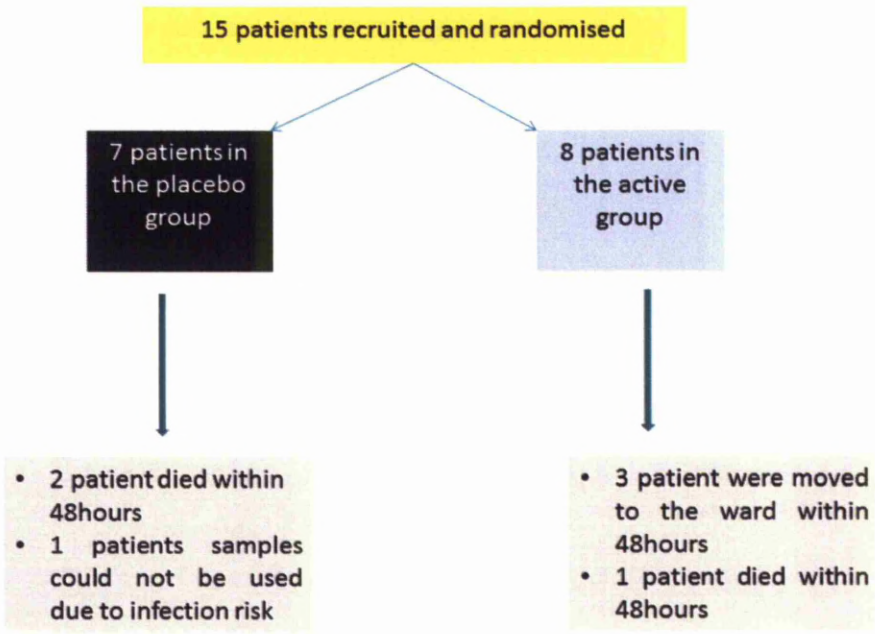


Figure 4.3: Illustration of the randomisation of patients for the clinical trial and the clinical outcome during their critical care stay.

4.2.4. Sample collection and analysis.

Baseline blood and muscle samples were obtained and according to randomisation and intravenous glutamine or iso-caloric glucose was administered. Further samples were taken at 48hours and 96hours from randomisation. Blood samples were centrifuged at 1500g for 5min. Serum and

plasma was stored at -80^{0}C. Muscle samples obtained (~50 mg) were immediately frozen in liquid nitrogen and stored at –80°C for later analysis (Section 2.1.2.2.2.1; Section 2.1.2.2.2.2).

4.2.5 Statistical analysis

Statistical analysis was carried out with Statistical Package for Social Sciences (SPSS) software version 15. Student's *t*-tests were carried out to analyse differences between two groups. Where multiple comparisons were made, data were analysed with ANOVA and accounted for repeated measures where necessary. When a significant F-value was observed; post-hoc analysis was performed to identify significance where appropriate. Significance was set at the level of ≤ 0.05. Data are presented as means ± standard error of the mean (SEM).

4.3. Results

4.3.1. Demographics

Figure 4.4 illustrates the age in years of the placebo and glutamine treated groups respectively. The mean age of the placebo treated group was 61years with a range from 37 – 83years. 37.5% were women. The mean age of the glutamine treated group was 66.5years with a range from 46 – 92years. 42.85% were women. There was no significant difference between the age and gender of the two groups.

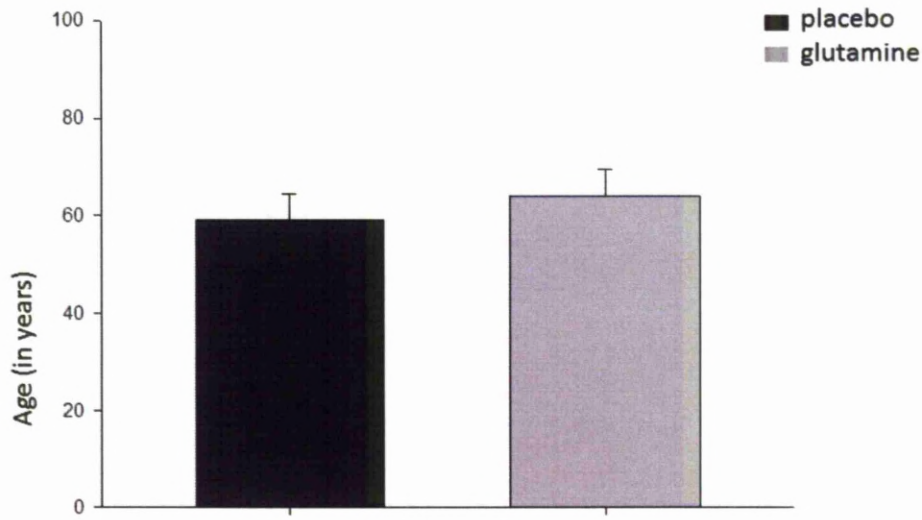


Figure 4.4: Age in years in placebo and glutamine group. Data presented as mean in years +/- SEM, n=7.

Figure 4.5 shows the APACHE II score at time of randomisation (Section 1.1.1). Mean APACHE II score of the placebo treated group was 19.14 +/- 2.37 (SEM), range [12 – 29]. Mean APACHE II score of the glutamine treated group was 15.29 +/- 1.45 (SEM), range [11 – 22]. Data demonstrated no significant difference between the APACHE II score of the placebo treated group compared with the glutamine treated group.

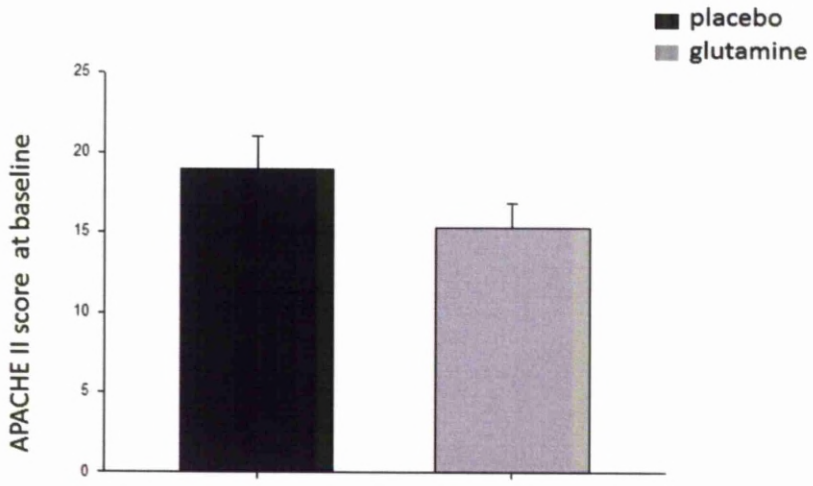


Figure 4.5: APACHE II score at time of randomisation in placebo and glutamine group. Data presented as mean +/- SEM, n=7.

4.3.2 Primary Outcomes

4.3.2.1 HSP 70 concentration in serum of patients

Figure 4.6 shows the serum HSP 70 content at three predefined time points. Data demonstrate no significant difference between the serum HSP 70 content of the placebo treated group compared with the glutamine treated group at any time point.

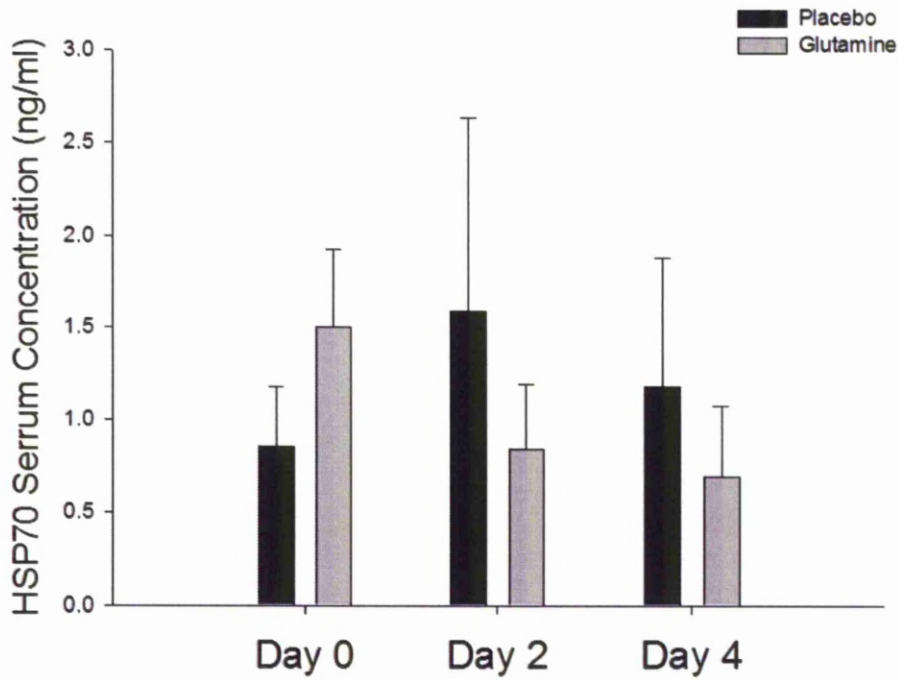


Figure 4.6: Serum highly inducible HSP 70 content of patients treated with either placebo or glutamine at baseline, 48hours and 96hours post-initialisation of study. Data presented as mean +/- SEM; n=4.

4.3.2.2 Heat shock protein content of muscle of patients

Figure 4.7 shows the muscle HSP 70 content at three predefined time points. Data demonstrate no significant difference between the muscle HSP 70 content in the placebo treated group compared with the glutamine treated group.

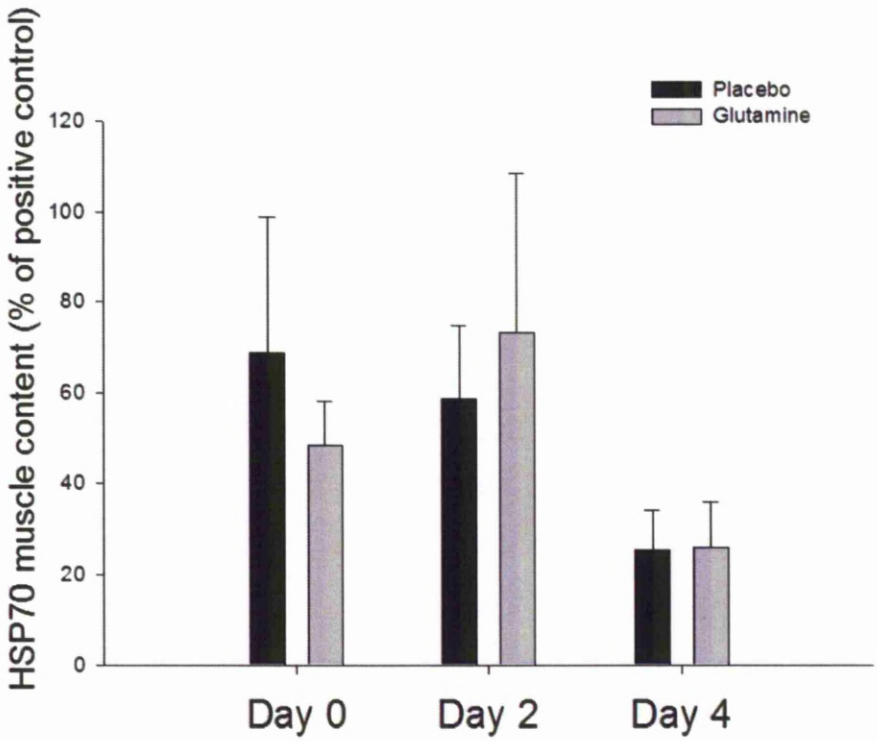


Figure 4.7: Muscle HSP 70 content of patients treated with either placebo or glutamine at baseline, 48hours and 96hours post- initialisation of study. HSP 70 content is expressed as a percentage of positive control. Data presented as mean +/- SEM, n=4.

Figure 4.8 shows the muscle HSC 70 content at three predefined time points. Data demonstrated no significant difference between the muscle HSC 70 content in the placebo treated group compared with the glutamine treated group.

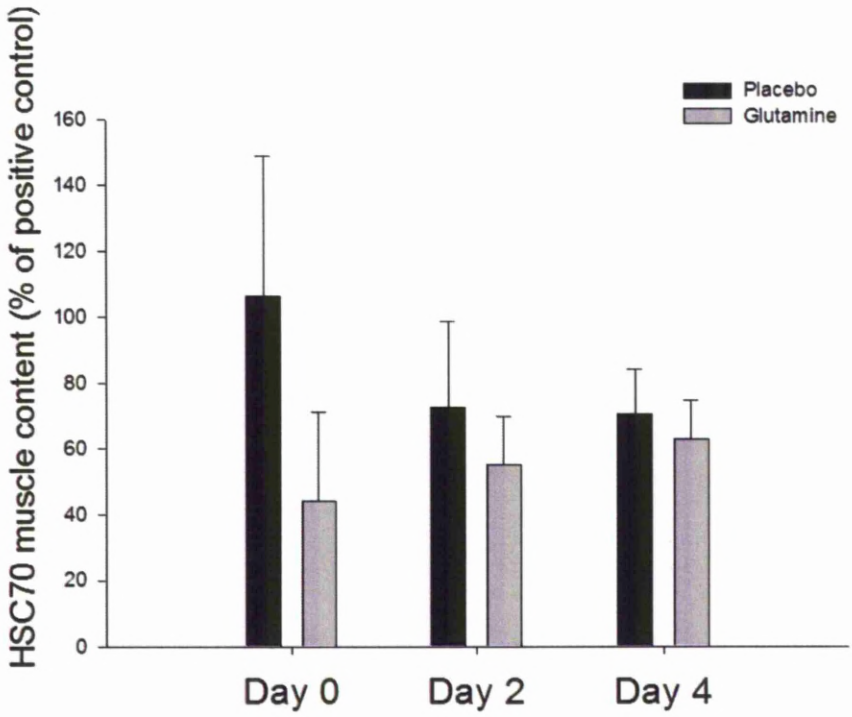


Figure 4.8: Muscle HSC 70 content of patients treated with either placebo or glutamine at baseline, 48hours and 96hours post- initialisation of study. HSC 70 content is expressed as a percentage of positive control. Data presented as mean +/- SEM, n=4.

Figure 4.9 shows the muscle HSP 60 content at three predefined time points. Data demonstrated no significant difference between the muscle HSP 60 content in the placebo treated group compared with the glutamine treated group.

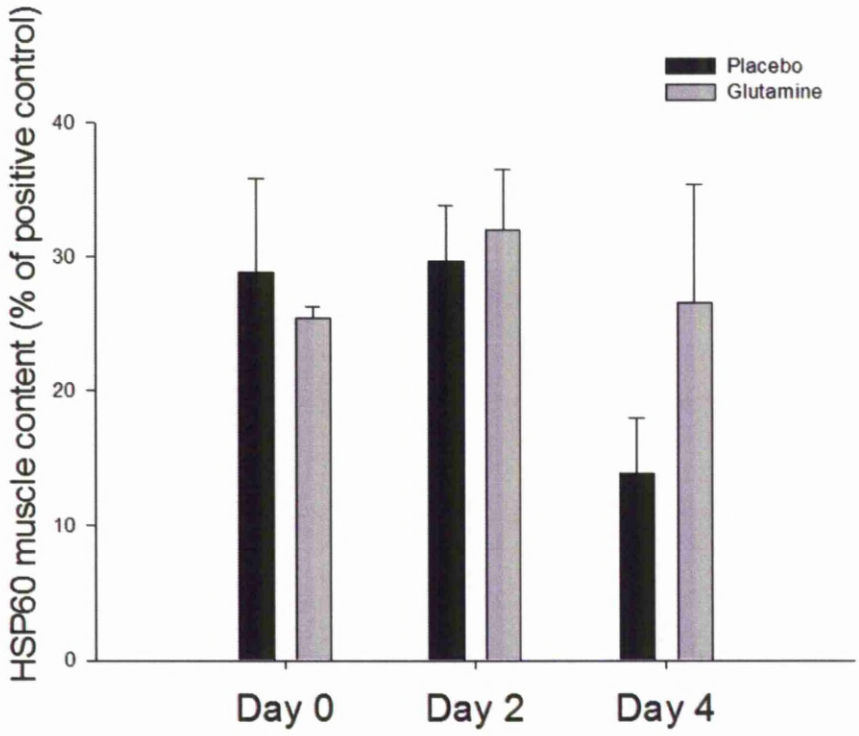


Figure 4.9: Muscle HSP60 content of patients treated with either placebo or glutamine at baseline, 48hours and 96hours post- initialisation of study. HSP 60 content is expressed as percentage of positive control. Data presented as mean +/- SEM, n=4 (see Figure 4.3).

Figure 4.10 shows the muscle α B crystallin concentration at three predefined time points. Data demonstrated no significant difference between the muscle α B crystallin content in the placebo group compared to the glutamine group. α B crystallin content expressed in percentage from positive control. Data presented as mean +/- SEM, n=4.

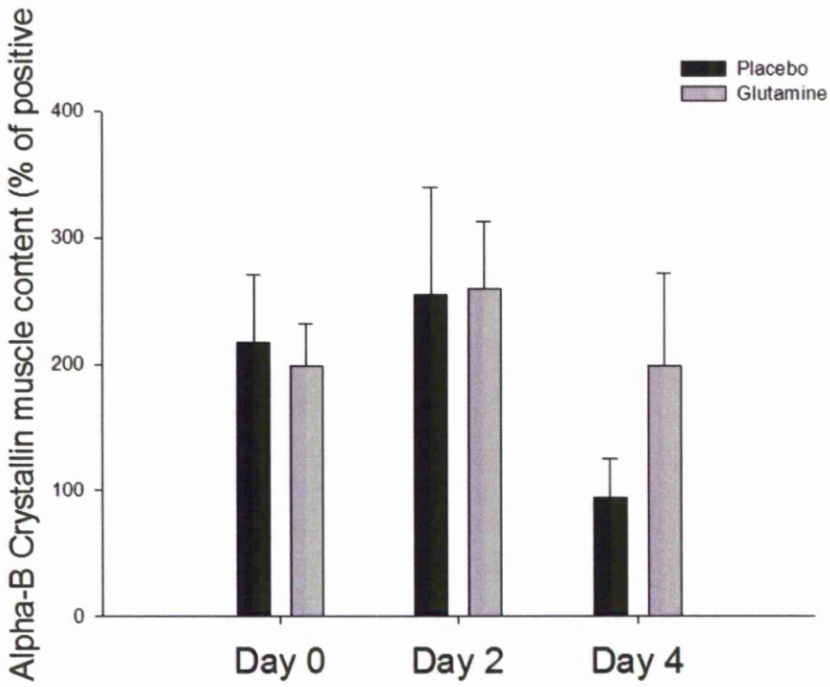


Figure 4.10: Muscle α B crystallin content of patients treated with either placebo or glutamine at baseline, 48hours and 96hours post- initialisation of study. α B crystallin content expressed in percentage of positive control. Data presented as mean +/- SEM, n=4 (see Figure 4.3).

Figure 4.11 shows the muscle HSP 10 content at three predefined time points. Data demonstrate no significant difference between the muscle HSP 10 content in the placebo treated group compared with the glutamine treated group.

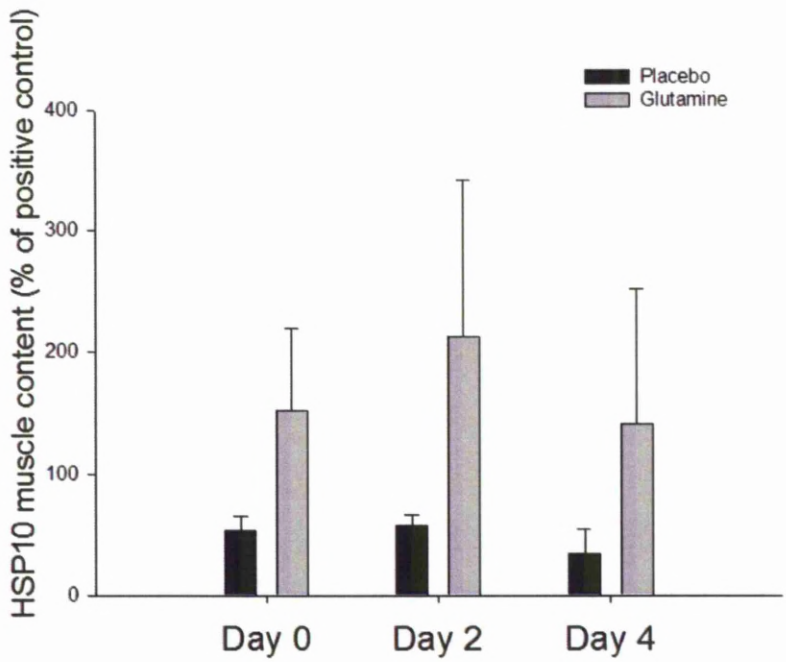


Figure 4.11: Muscle HSP 10 content of patients treated with either placebo or glutamine at baseline, 48hours and 96hours post- initialisation of study. HSP 10 content is expressed as percentage of positive control. Data presented as mean +/- SEM, n=4 (see Figure 4.3).

4.3.2.3 Cytokine content of serum

Figure 4.12 and 4.13 show the serum concentration of TNF-α and IL 6 at three predefined time points. Data demonstrated no significant difference between the serum TNF-α or IL 6 content of the placebo treated group compared with the glutamine treated group although there appears to be a trend towards

maintenance of TNF-α levels in the glutamine treated group. Data presented as mean +/- SEM, n=4.

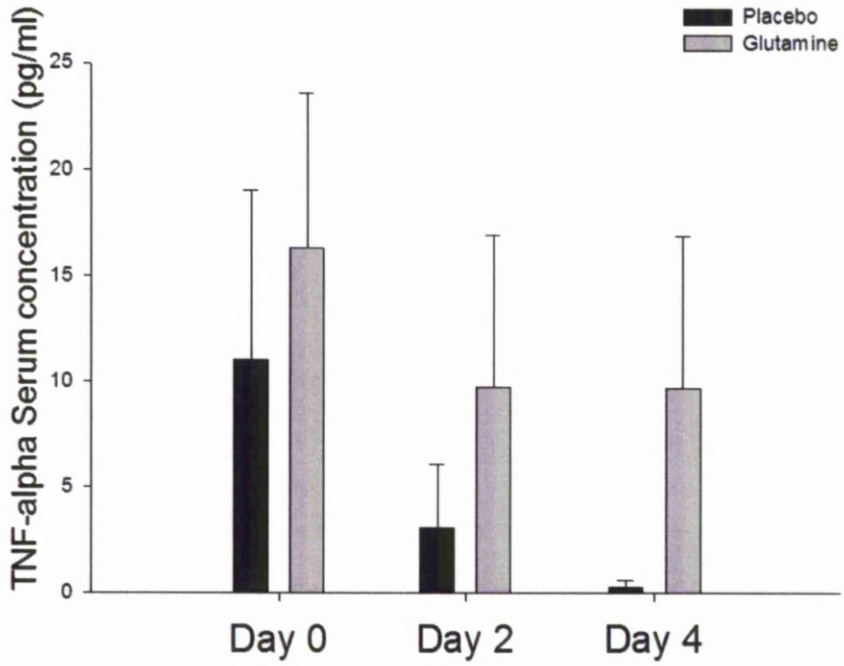


Figure 4.12: TNF-α concentration in serum at baseline, 48hours and 96hors following randomisation into placebo treated or glutamine treated group. TNF-α is expressed in pg/ml. Data are presented as mean +/- SEM, n=4.

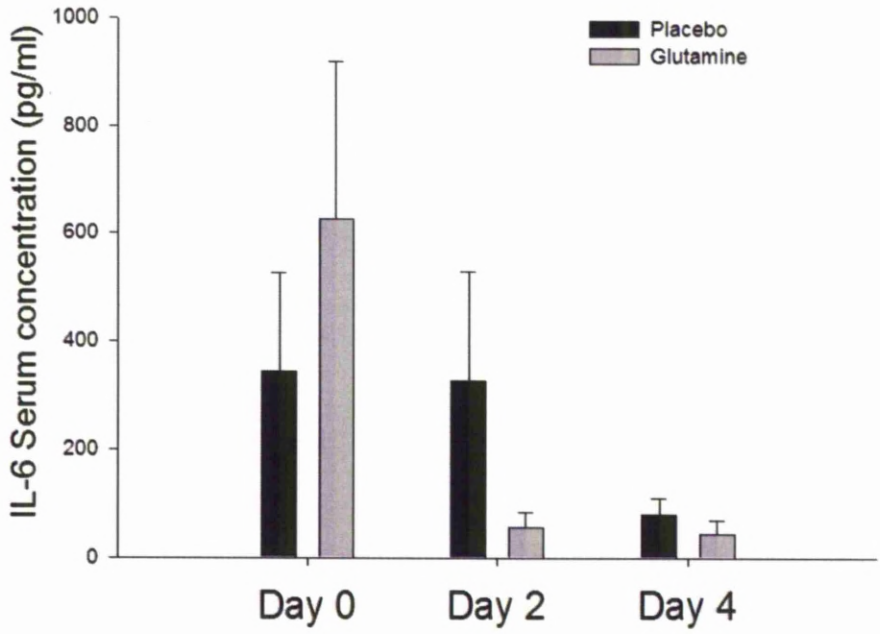


Figure 4.13: IL 6 concentration in serum at baseline, 48hours and 96hors following randomisation into placebo treated or glutamine treated group. IL 6 concentration is expressed in pg/ml. Data are presented as mean +/- SEM, n=4.

4.3.2.4 Glutamine concentration in plasma

Figure 4.14 and Table 4.1 show the plasma concentration of glutamine at the three predefined time points. Data demonstrated no significant difference between the plasma glutamine content in the placebo treated group compared with the glutamine treated group. Glutamine content is expressed in mmol/l. Data presented as mean +/- SEM, n=4.

	Placebo		Glutamine	
	Mean (mmol/l)	SEM (mmol/l)	Mean (mmol/l)	SEM (mmol/l)
Day 0 n=7	0.567	0.075	0.539	0.0589
Day 2 n=4	0.589	0.066	0.63	0.131
Day 4 n=4	0.536	0.103	0.65	0.134

Table 4.1: Glutamine concentration in plasma at baseline, 48hours and 96hours following randomisation into the placebo treated or glutamine treated group. Glutamine concentration is expressed in mmol/l. Data presented as mean +/- SEM, n=4.

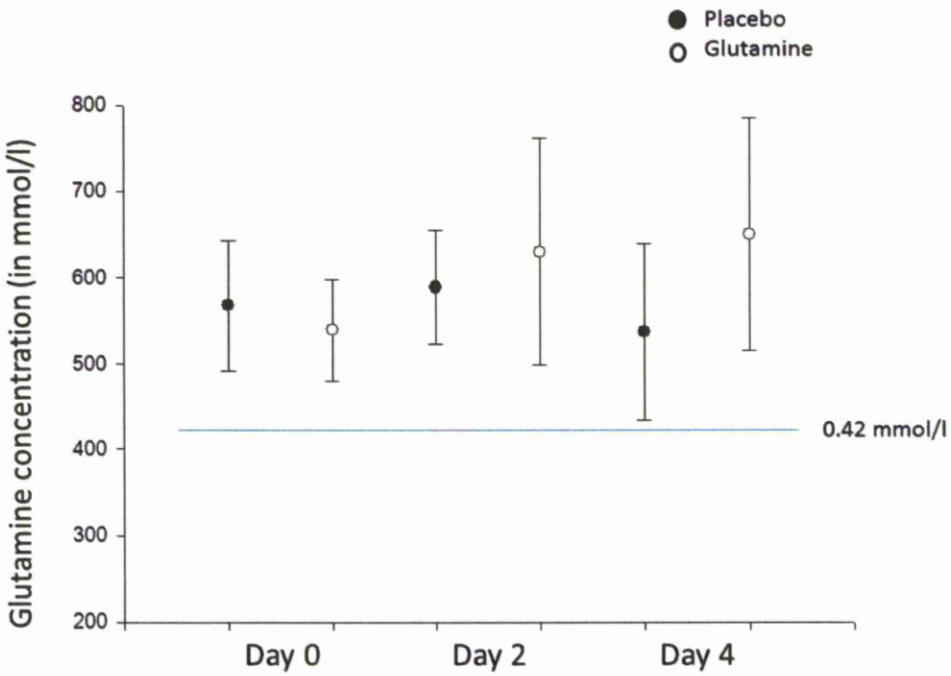


Figure 4.14 Glutamine concentration in plasma at baseline, 48hours and 96hours following randomisation into placebo treated or glutamine treated group. Glutamine concentration is expressed in mmol/l. Data presented as mean +/- SEM, n=4. (Blue line represents 0.42 mmol/l. ≥

0.42mmol/l glutamine concentration in plasma is considered normal (Oudermans-van Straaten et al. 2001).

4.3.2.5 Correlation of plasma glutamine concentration and serum HSP 70 concentration.

Figure 4.15 shows the correlation between serum HSP 70 concentration and plasma glutamine concentration in patients from both treatment groups at 96hours following randomisation. The x-axis represents the serum HSP 70 concentration; the y-axis represents the corresponding plasma glutamine concentration. Data demonstrate no significant correlation between the plasma glutamine content and the serum HSP 70 concentration.

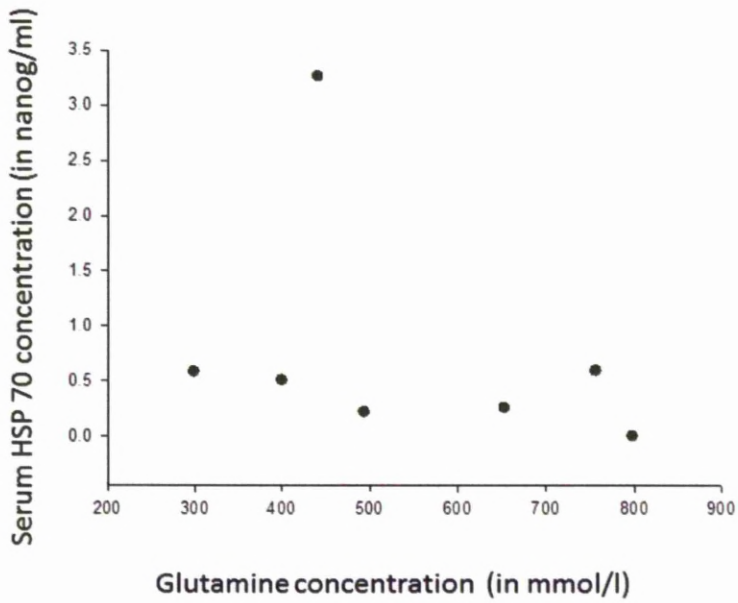


Figure 4.15 Correlation between plasma glutamine concentration and serum HSP 70 concentration at 96hours following randomisation.

4.3.2.6 Correlation of plasma glutamine concentration and muscle HSP content.

Figure 4.16 shows the correlation between muscle HSP 70 content and plasma glutamine concentration in patients from both treatment groups at 96hours following randomisation. Data demonstrate a weak correlation between the plasma glutamine concentration and muscle HSP 70 content.

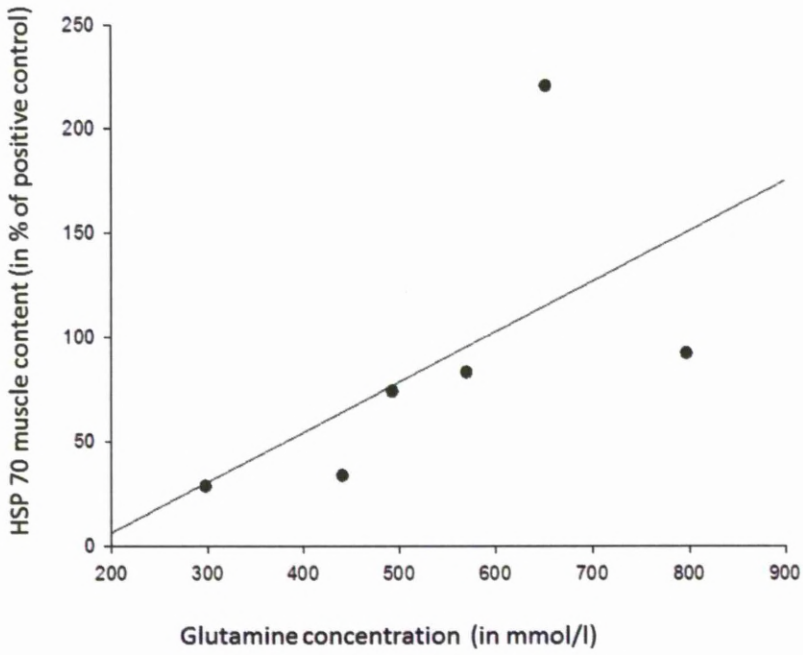


Figure 4.16 Correlation between plasma glutamine concentration and muscle HSP 70 content at 96hours following randomisation. Correlation coefficient r=0.601.

Figure 4.17 shows the correlation of muscle HSC 70 content and plasma glutamine concentration in patients from both treatment groups at 96hours from randomisation. Data demonstrate a weak correlation between the plasma glutamine concentration and muscle HSC 70 content.

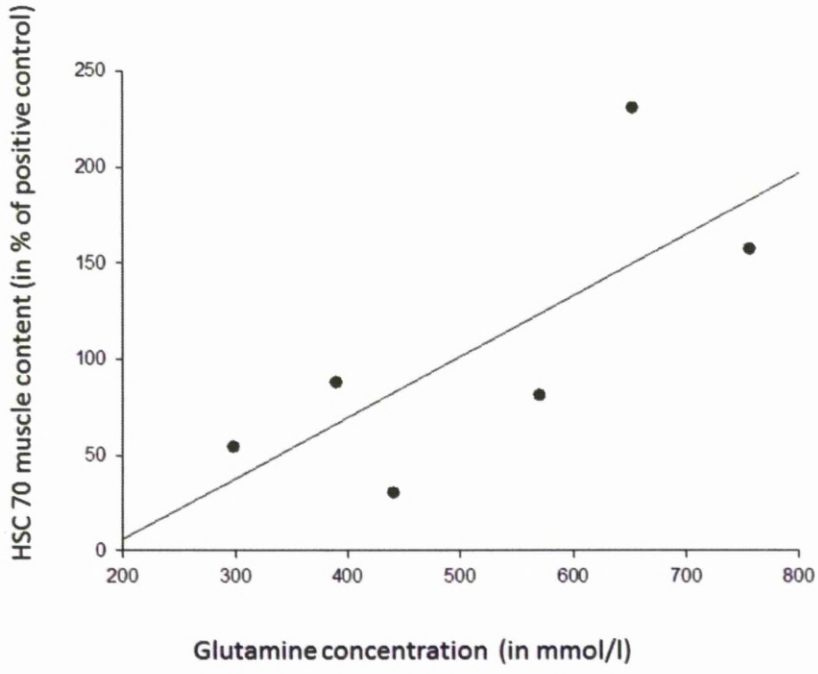


Figure 4.17 Correlation between plasma glutamine concentration and muscle HSC 70 content at 96hours following randomisation. Correlation coefficient r=0.738.

Figure 4.18 shows the correlation of muscle HSP 60 content and plasma glutamine concentration in patients from both treatment groups at 96hours from randomisation. Data demonstrate no correlation between the plasma glutamine concentration and muscle HSP 60 content.

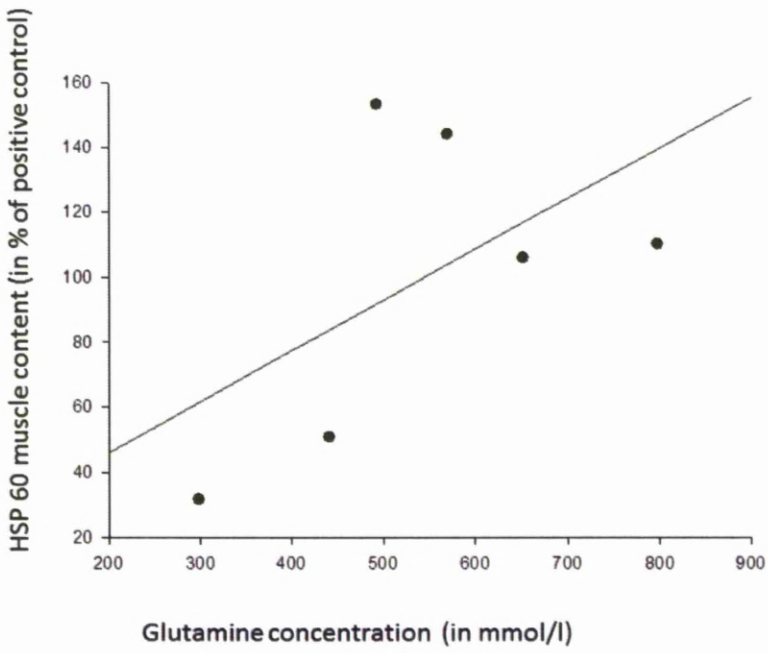


Figure 4.18: Correlation between plasma glutamine concentration and muscle HSP 60 content at 96hours following randomisation. Correlation coefficient r=0.552.

Figure 4.19 shows the correlation of muscle α B crystallin content and plasma glutamine concentration in patients from both treatment groups at 96hours from randomisation. Data demonstrate a significant correlation between the plasma glutamine concentration and muscle α B crystallin content.

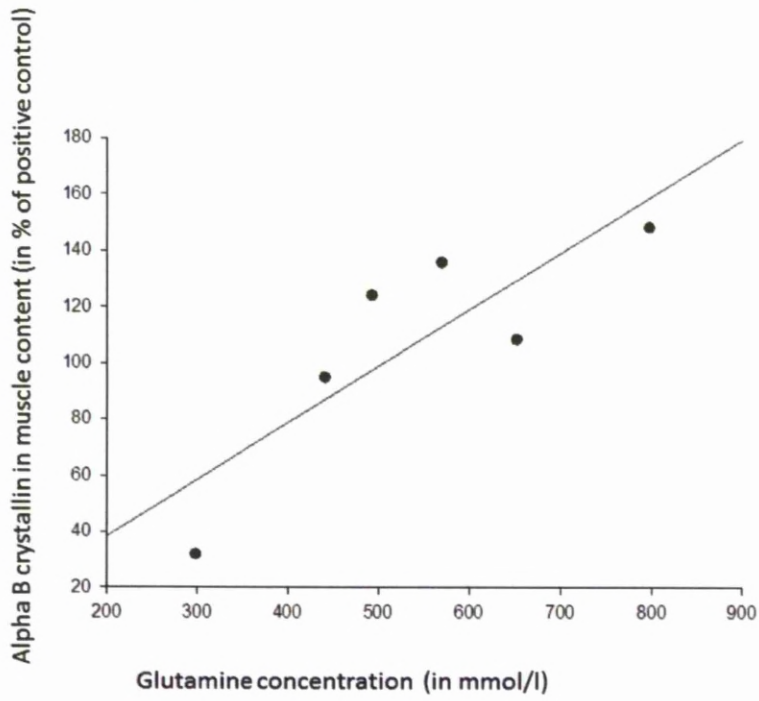


Figure 4.19 Correlation between plasma glutamine concentration and muscle α B crystallin concentration at 96hours following randomisation. Correlation coefficient r=0.839.

Figure 4.20 shows the correlation of muscle HSP 10 content and plasma glutamine concentration in patients from both treatment groups at 96hours from randomisation. Data demonstrate a strong correlation between the plasma glutamine concentration and muscle HSP 10 content.

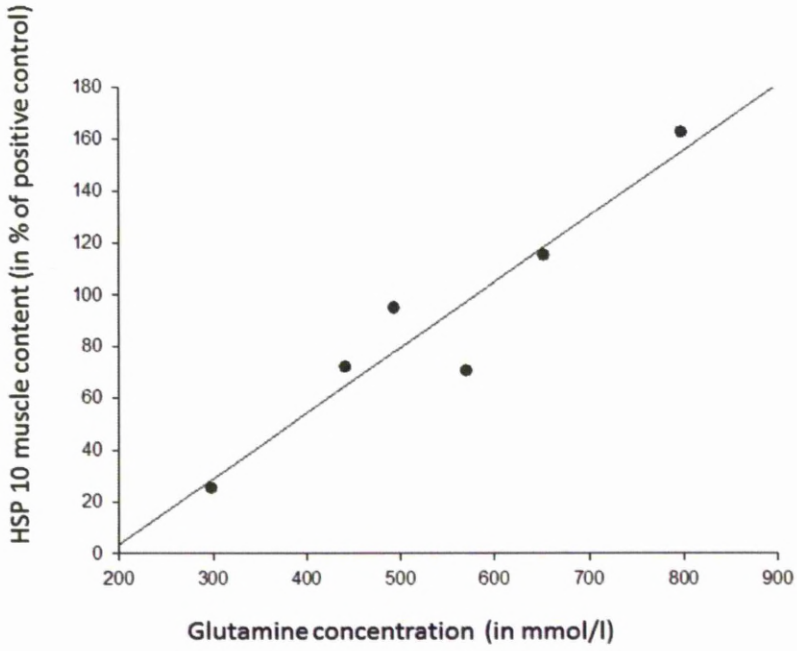


Figure 4.20 Correlation between plasma glutamine concentration and muscle HSP 10 content at 96hours following randomisation. Correlation coefficient r=0.945.

4.3.2.7 Glutathione

4.3.2.7.1. Glutathione content of muscle

Figures 4.21 and 4.22 show the muscle content of oxidised and total glutathione at three predefined time points. Data demonstrated no significant difference between the muscle content of oxidised and total glutathione in the placebo treated group compared with the glutamine treated group.

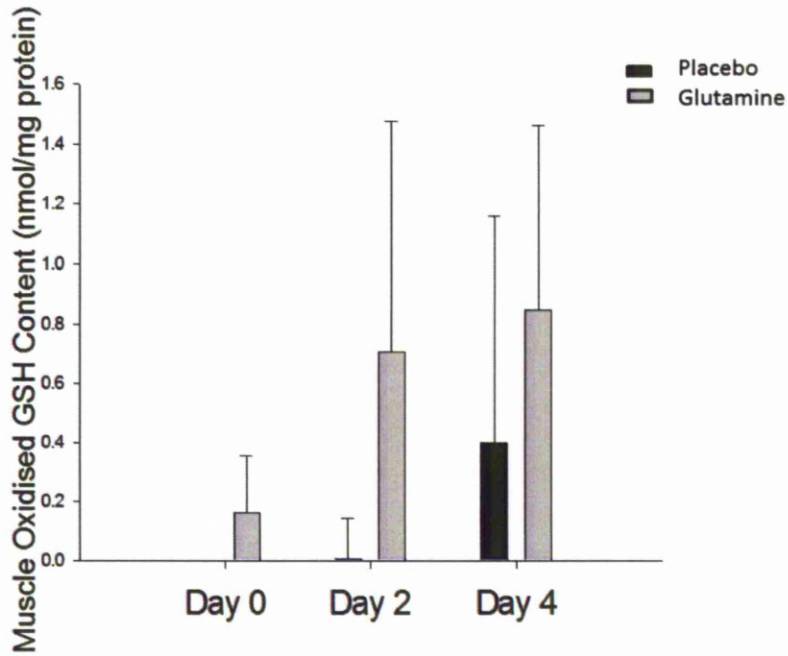


Figure 4.21 Oxidised glutathione content of muscle at baseline, 48hours and 96hours following randomisation into placebo treated or glutamine treated group. Data presented as mean +/- SEM, n=4.

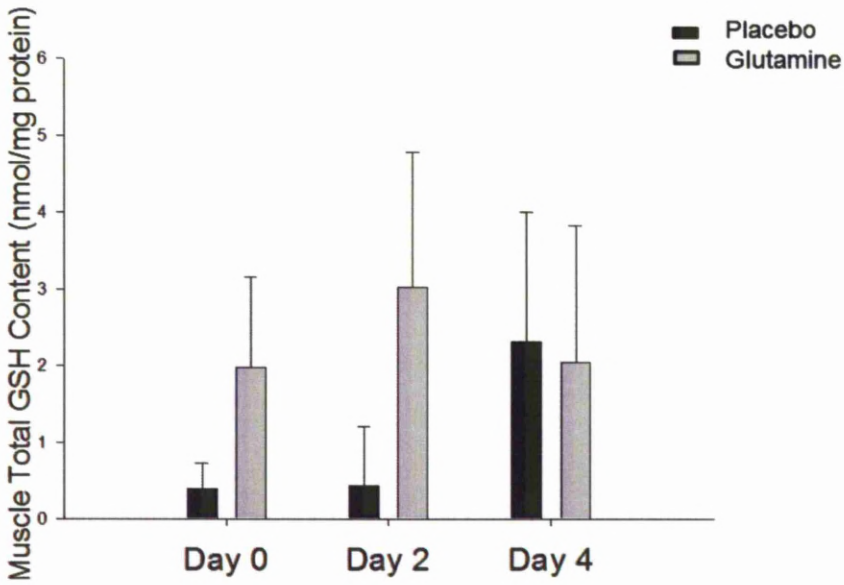


Figure 4.22 Total glutathione content of muscle at baseline, 48hours and 96hours following randomisation into placebo treated or glutamine treated group. Data presented as mean +/- SEM, n=4 (see Figure 4.3).

4.3.2.7.2. Glutathione concentration in plasma

Figures 4.23 and 4.24 show the plasma concentration of oxidised and total glutathione at three predefined time points. Data demonstrated no significant difference between the plasma content of oxidised and total glutathione in the placebo treated group compared with the glutamine treated group.

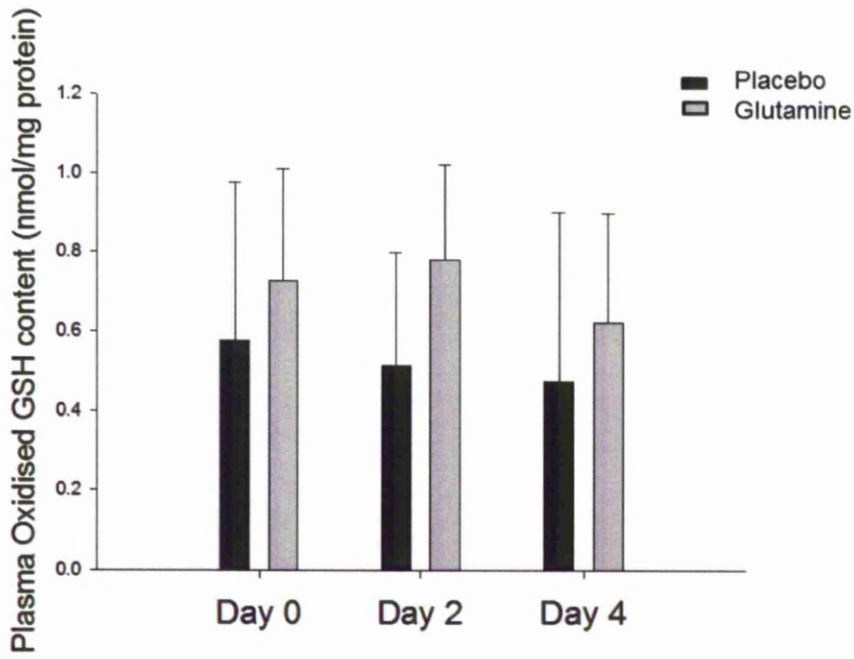


Figure 4.23 Oxidised glutathione concentration of plasma at baseline, 48hours and 96hours following randomisation into placebo treated or glutamine treated group. Data presented as mean +/- SEM, n=4.

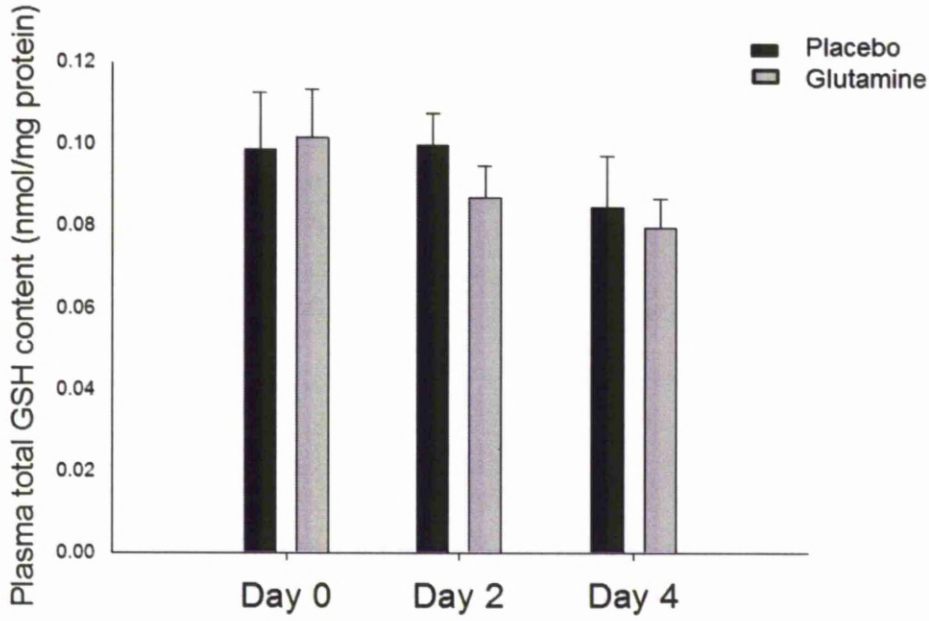


Figure 4.24 Total glutathione content of plasma at baseline, 48hours and 96hours following randomisation into placebo treated or glutamine treated group. Data presented as mean +/- SEM, n=4.

4.3.3 Secondary Outcomes

4.3.3.1. Illness Severity scores used at baseline, 48hours and 96hours.

Figure 4.25 shows the APACHE II score at three predefined time points. Mean APACHE II score of the placebo treated group was 19, range [12-29], 13.67 [3-21] and 15.8 [7-26]. Mean APACHE II score of the glutamine treated group was 15.29, range [11-22], 16.29 [2-30] and 10.5 [2-21]. Data demonstrated no significant difference between the APACHE II scores of the placebo treated group compared with the glutamine treated group.

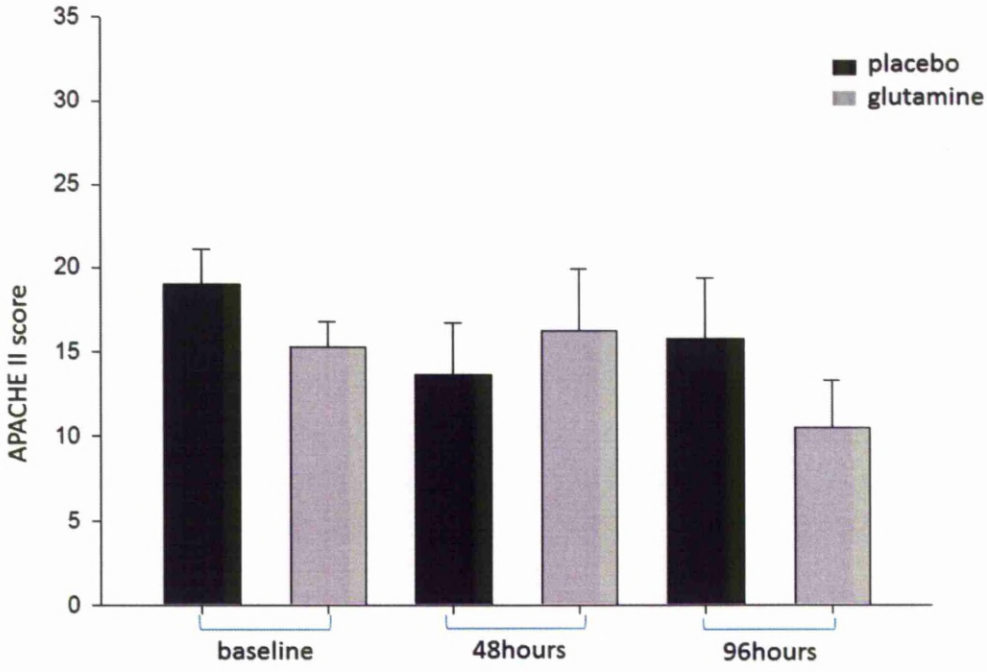


Figure 4.25 APACHE II scores at baseline, 48hours and 96hours. Data presented as mean +/- SEM, n=4.

Figure 4.26 shows the Simplified Acute Physiology Score (SAPS) at three predefined time points. Mean SAPS score of the placebo was 43, range [26-69], 35.2 [15-54] and 39.2 [18-64]. Mean SAPS score of the glutamine group was 38.7, range [30-60], 35.4 [18-60] and 27.2 [13-55]. Data demonstrated no significant difference between the SAPS scores of the placebo treated group compared with the glutamine treated group.

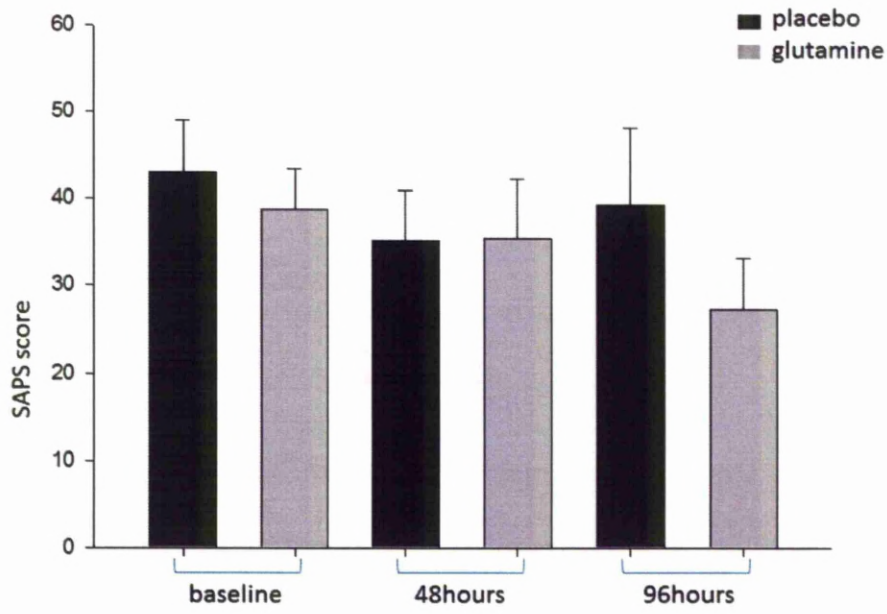


Figure 4.26 Simplified Acute Physiology Score (SAPS) at baseline, 48hours and 96hours. Data presented as mean +/- SEM, n=4.

Figure 4.27 shows the Sequential Organ Failure Assessment (SOFA) score at three predefined time points. Mean SOFA score of the placebo treated group was 9, range [6-12], 6.8 [2-10] and 5.2 [3-7]. Mean SAPS score of the glutamine treated group was 5.6, range [1-12], 5.9 [0-19] and 2.5 [0-10]. Data demonstrated no significant difference between the SOFA scores of the placebo treated group compared with the glutamine treated group.

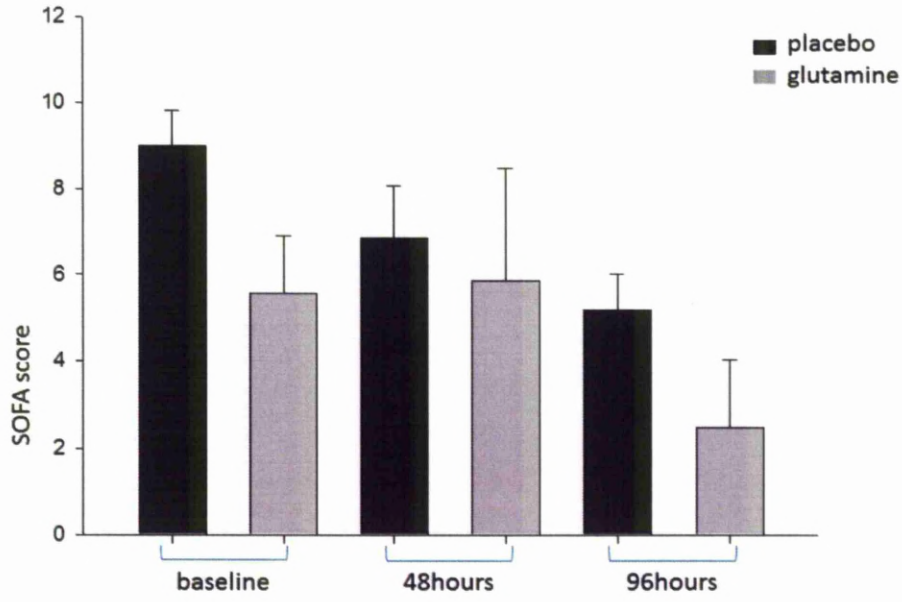


Figure 4.27 Sequential Organ Failure Assessment (SOFA) score at baseline, 48hours and 96hours. Data presented as mean +/- SEM, n=4.

4.3.3.2. Serum inflammatory markers in patients in the placebo and glutamine – treated groups over the first 96hours following randomisation.

Figure 4.28 shows the maximal body temperature in 24hour periods starting from 24hours before randomisation up to 96hours after randomisation. Baseline temperature was recorded as the maximum temperature in the 24hour period. Data demonstrated no significant difference between the temperatures of the placebo treated group compared with the glutamine treated group.

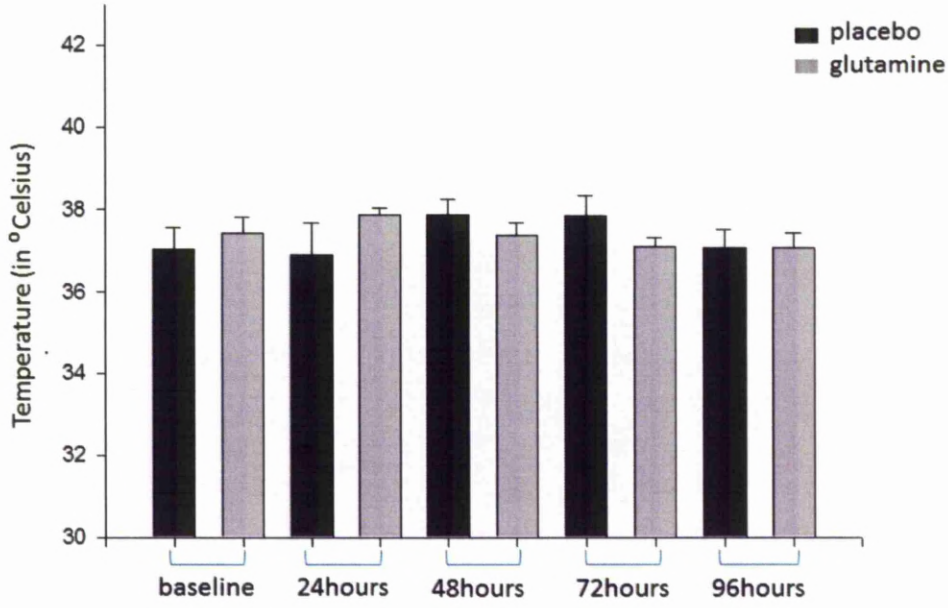


Figure 4.28 Body temperature at baseline, 24hours, 48hours, 72hours and 96hours following randomisation. Temperature expressed in °Celsius. Data presented as mean +/- SEM; n=4.

Figure 4.29 shows the white cell (WCC) count in full blood at five predefined time points. Data demonstrated no significant difference between the WCC of the placebo treated group compared with the glutamine treated group.

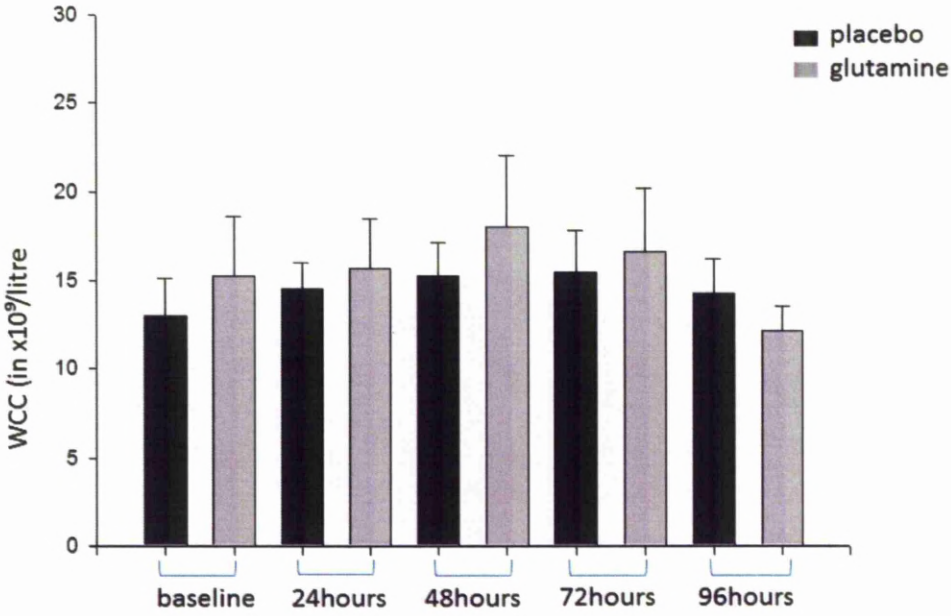


Figure 4.29 White Cell Count at baseline, 24hours, 48hours, 72hours and 96hours following randomisation. WCC expressed as white cells x 10^9/litre. Data presented as mean +/- SEM, n=4.

Figure 4.30 shows the concentration of the C-reactive protein (CRP) in serum at three predefined time points. Data demonstrated no significant difference between the CRP of the placebo treated group compared with the glutamine treated group. The graph further illustrates the initial worsening during critical illness followed by recovery of the patients that survived critical illness.

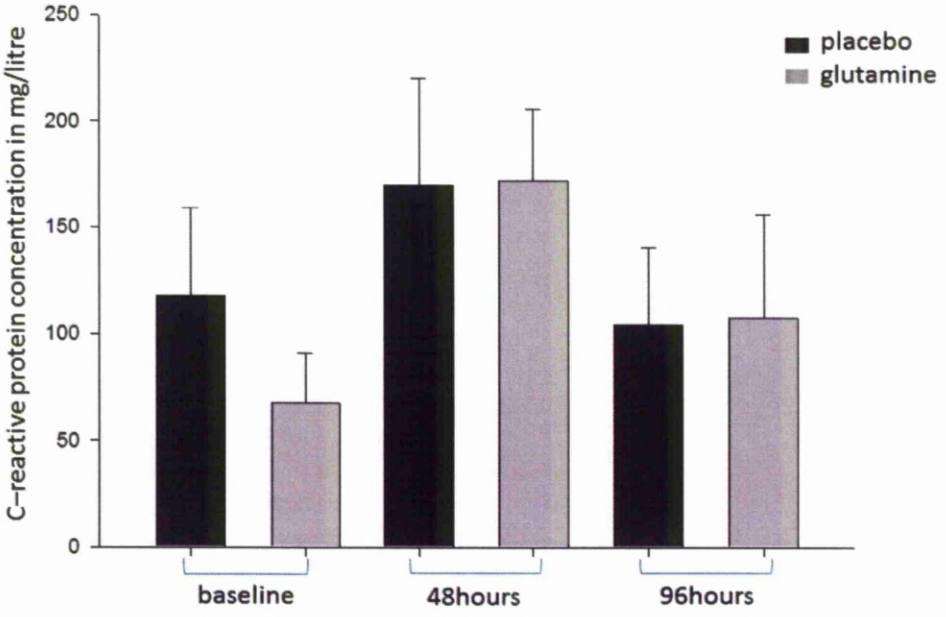


Figure 4.30 C-reactive protein at baseline, 48hours and 96hours from randomisation. CRP expressed in mg/litre. Data presented as mean +/- SEM, n=4.

4.3.3.3 Length of ventilation

Figure 4.31 shows the length of ventilation of patients in hours. Length of ventilation was defined as the time from intubation to either extubation or death. Data demonstrated no significant difference between the length of ventilation of the placebo treated group compared with the glutamine treated group.

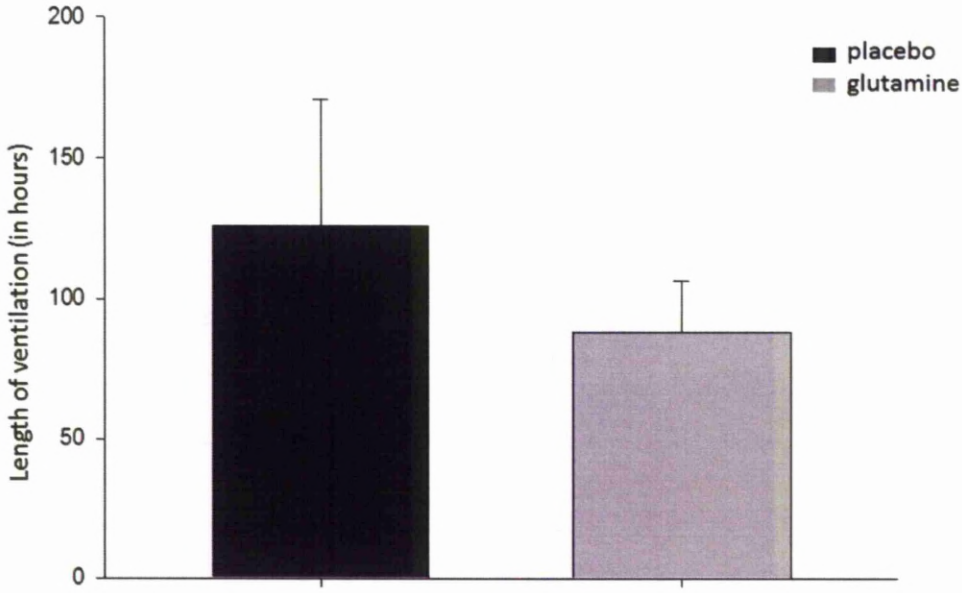


Figure 4.31 Length of ventilation. Length of ventilation expressed in hours. Data presented as mean +/- SEM, n=7.

4.3.3.4 Length of stay in critical care

Figure 4.32 shows the length of stay in critical care. Length of stay in critical care was defined as the time from admission to either discharge or death. Data demonstrated no significant difference between the length of stay on critical care of the placebo group compared to the glutamine group.

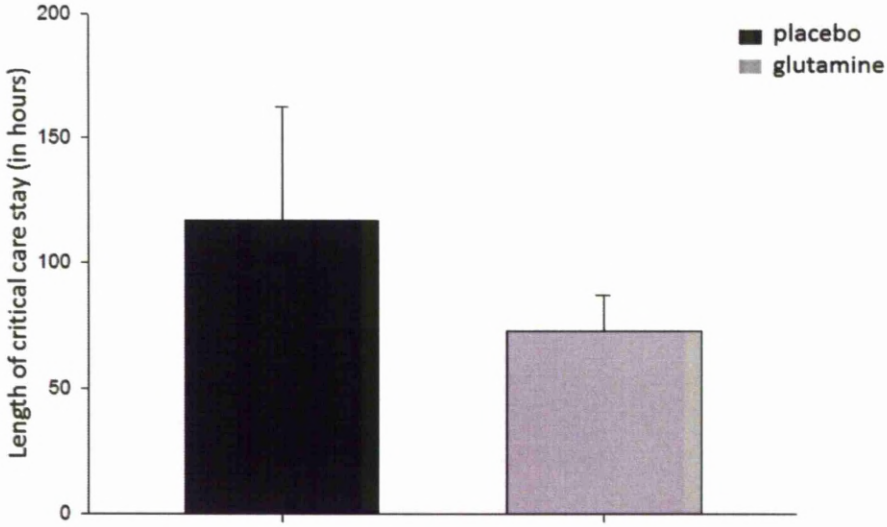


Figure 4.32 Length of stay on critical care. Length of stay on critical care expressed in hours. Data presented as mean +/- SEM, n=7.

4.3.3.5.Critical care mortality

Figure 4.33 shows the critical care survival. Critical care mortality was defined as death while on critical care. Data presented as percentage of patients alive in individual groups with 4/8 (50%) in the placebo treated group and 6/7 (85.7%) in the glutamine treated group.

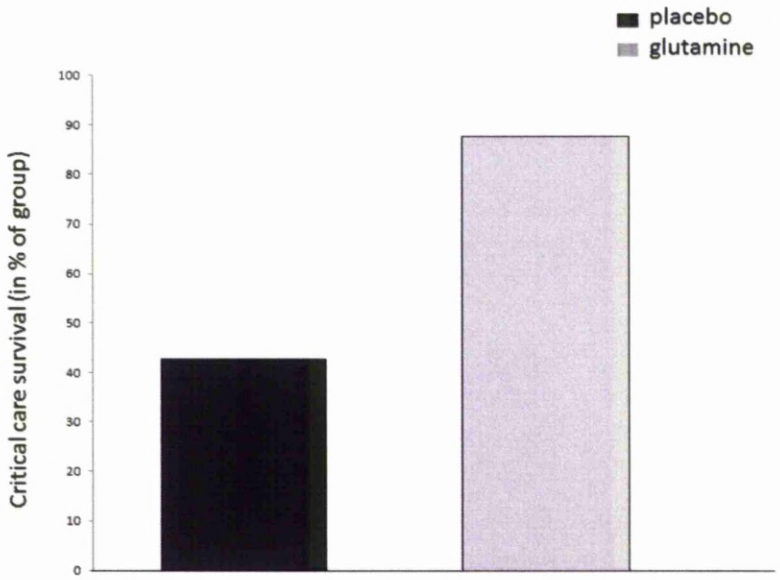


Figure 4.33 Critical Care survival. Data presented as percentage of patients alive in individual groups.

4.4. Discussion

Glutamine has been implicated to augment cell survival against a variety of stressful stimuli. This beneficial effect has been suggested to involve increased intracellular and extracellular HSP 70 concentrations (Wischmeyer et al. 1997; Musch et al. 1998; Ziegler et al. 2005) and indeed, intravenous glutamine administration has been shown to correlate with an increased concentration of intracellular and extracellular HSP 70 (Ziegler et al. 2005). Moreover a dose dependant response was observed following glutamine administration (Wischmeyer et al. 2001). This observation supported the view that glutamine has the ability to work as a "pharmacological regulator of HSP expression" facilitating higher intracellular HSP concentrations the higher the administrated glutamine dose (Wischmeyer et al. 2006).

The above described clinical study investigated the hypothesis that early, high dose intravenous glutamine supplementation will result in an increased HSP content of muscle and serum in the critically ill patient.

Despite previous reports of significantly increased HSP 70 content of serum and tissues after intravenous glutamine administration (Ziegler et al. 2005; Wischmeyer et al. 2001), no statistically significant difference could be observed between the two groups in either of the primary outcome measures (HSPs in serum and muscle, glutamine concentration in serum) in the above study.

Nonetheless, no significant change of HSP concentration after one large dose of intravenous glutamine was observed in either serum or muscle tissue. The dose of glutamine used in the clinical trial (0.5g/kg bodyweight) was indeed not dissimilar to the glutamine dose used in an animal sepsis model were a range of doses from 0g/kg bodyweight to 0.75g/kg bodyweight were used (Wischmeyer et al. 2001). Indeed even doses of 0.15g/kg bodyweight of glutamine did cause a significant HSP 72 rise. This observation raised the question as to whether the plasma glutamine concentration influences the HSP content in serum and muscle. Indeed, there appears to be a correlation between plasma glutamine concentration and muscle content of HSC 70, αB crystallin and HSP 10.

Several problems were encountered whilst undertaking this study. Firstly, the study was designed as a pilot study, as power calculations were not possible. It was therefore decided to recruit 30 patients (Lancaster et al. 2004). Clinical trials not infrequently encounter problems with recruitment despite the identification of sufficient numbers of patients (Prescott et al. 1999) and indeed this study could only recruit 15 patients in the given time available (June 2006 – July 2007) which had a significant impact on the end result. Further, as outlined in Figure 4.3, data was incomplete in 7 patients for reasons outlined.

One inclusion criterion was an APACHE II score of > 10 to guarantee the recruitment of patients who were significantly ill. The mean APACHE II in treatment and placebo group was 15 and 19, which compared with previous studies with APACHE II scores of 13 (Ziegler et al. 2005) and 17 (Griffiths et

al. 1997) respectively in the treatment groups. "Need for parenteral nutrition/ gastrointestinal failure" was a further inclusion criterion for both trials (Griffiths et al. 1997; Ziegler et al. 2005) not used in this clinical trial. The inclusion criterion of "need for parenteral nutrition/ gastrointestinal failure", an independent risk factor for critical care mortality (Reintam et al. 2006), selects patients who are potentially more ill than patients without gastrointestinal failure. Further, critical care patients are a rather heterogeneous population (Carson and Shorr. 2003). Pre – selection of patients with "need for parenteral nutrition/ gastrointestinal failure" might therefore reduce the heterogeneity of the study group (Carson and Shorr. 2003).

Rat sepsis models using an endotoxaemia sepsis model (Wischmeyer et al. 2001) and a caecal ligation sepsis model (Singleton et al. 2005) have shown a mortality benefit with an increased intracellular HSP 70 content of several tissues of the rat after one large intravenous glutamine dose administered after the septic insult. However, the glutamine dose was infused within minutes of the septic insult rather than ~ 24hours after admission to critical care as in our study and again the animal sepsis models did not suffer from critical care heterogeneity (Carson and Shorr. 2003).

In a human endotoxaemia model of healthy volunteers, glutamine at a dose of 0.25g/ kg bodyweight and endotoxin were administered intravenously. Plasma glutamine concentration increased but no change in plasma TNF-α or IL 6 was observed with intravenous glutamine, neither did the HSP content of blood mononuclear cells change (Andreasen et al. 2009)

Surprisingly, the measured plasma glutamine concentrations in our study were relatively normal and only three patients had glutamine concentration below 0.4mmol/l at the time of randomisation whereas glutamine concentrations are found to correlate with mortality (Rodas et al. 2012).

The results of the clinical study do not suggest an effect above the correction of a deficiency. Further, the glutamine dose used was significantly large, however only administered once. Considering the length of stay of an average of > 4 days on critical care, the cumulative dose was considerably smaller than previous studies used (Ziegler et al.2005).

The results therefore need to be interpreted with caution. Noteworthy, one patient died in the glutamine treated group compared with 4 patients in the placebo treated group and, although not statistically significant, the placebo group appeared more severely ill (Mean+/- SEM 15.29 +/- 1.45 glutamine/ 19.14 +/- 2.37 placebo).

None the less, this study could not find any evidence to support the early use of one large single dose of intravenous glutamine.

CHAPTER 5

GENERAL DISCUSSION

5.1. Summary of findings of the work presented

5.1.1. The interaction of extracellular glutamine and HSPs

5.1.1.1. Cell culture analyses

- During differentiation, maturation is inhibited in low extracellular glutamine concentrations, below 0.5mM glutamine. Extracellular glutamine concentrations of 0.5mM and 1mM glutamine during differentiation correlated with increased intracellular HSP 70 content suggesting a stress response. Higher glutamine concentration did not affect intracellular HSP 70 content.
- The intracellular HSP content of myotubes at rest was not affected by modulation of extracellular glutamine concentrations after a period of relative deficiency.
- The intracellular HSP content of C2C12 myotubes grown in 25% of normal glutamine (0.5mM) was reduced following a period of heat stress compared with C2C12 myotubes grown in media deficient in glutamine (0.5mM) and then replete with standard (2mM) extracellular glutamine.
- The intracellular HSP content of C2C12 myotubes grown in 25% of normal glutamine (0.5mM) medium and subsequently replete with 2.5 x normal glutamine concentrations (5mM) and treated with TNF-α was increased in a similar manner to C2C12 myotubes grown in media deficient in glutamine (0.5mM) and then replete with standard (2mM) extracellular glutamine and treated with TNF-α.

5.1.1.2. During critical illness

- The clinical study did not show a significant change in plasma glutamine concentration at any of the time points measured after one large intravenous glutamine dose administered early in critical illness.

- The clinical study did not show a significant change in HSP content of either muscle or serum at any of the time points measured after one large intravenous glutamine dose administered early in critical illness.
- No significant difference was observed in any of the secondary outcome measures after one large intravenous glutamine dose administered early in critical illness.
- A striking correlation was observed between plasma glutamine concentration and muscle HSP 10 content and a significant correlation was observed between plasma glutamine concentration and muscle HSC 70 and α B crystallin content respectively.

5.1.2. Summary of interpretation of results

Higher than normal/sufficient (5mM) extracellular glutamine concentrations did not appear to influence the muscle intracellular content of the highly inducible HSP 70 compared with standard concentrations in a cell culture stress model. Similarly, one large dose of intravenous glutamine in critical illness did neither influence the content of HSPs in serum nor muscle. This is contrary to observations by other authors (Wischmeyer et al. 2001) who administered one dose of glutamine intravenously in a rat sepsis model where the glutamine amount given intravenously correlated with the HSP 70 content of various tissues of the rat and was greater with greater glutamine doses given (Wischmeyer et al. 2001). The observation, that glutamine administration in rat and mice sepsis models increases the HSP 70 content was reproduced recently (Zhao et al. 2012; Mazloomi et al. 2011). These effects were observed in glutamine doses of 0.75g/kg bodyweight (Wischmeyer et al. 2001; Mazloomi et al. 2011) and indeed significant peak plasma glutamine concentrations of > 2.5 times in the glutamine treatment group compared with the control group was observed (Wischmeyer et al. 2001).

Of course comparisons between our cell culture model and animal sepsis models need to be made with caution. The heat stress and the TNF-α stress in the cell culture model does not necessarily resemble stress during an animal

sepsis model. Further, although we tried to develop in house HPLC to be able to determine intracellular glutamine concentrations, this was not established between 2004 and 2007. We can therefore only assume that the extracellular glutamine concentrations correlate with the intracellular glutamine concentrations.

Maintenance of a moderate deficiency (to 25% of normal values; 0.5mM) of glutamine resulted in reduced HSP 70 content of heat stressed muscle cells compared with the HSP 70 content of heat stressed cells repleted into standard glutamine concentrations. This observation is in concordance with previous work showing a reduced HSP 70 content of tissues during glutamine deficiency (Oehler et al. 2002). The importance of a glutamine deficiency is further highlighted by the possible correlation between the plasma glutamine concentration and several muscle HSPs in the clinical trial suggesting the importance of the plasma glutamine concentration rather than the glutamine amount given at the time of critical illness. The cell culture experiments as well as the clinical trial do therefore highlight the effect of a glutamine deficiency on HSP content and the importance to correct a clinical deficiency to improve tissue protection.

We could not show a beneficial effect of one large single dose of intravenous glutamine early during critical illness. Neither could we show a significant increase in plasma glutamine concentrations 48hours and 96hours after one large dose of intravenous glutamine. In an animal sepsis model (Wischmeyer et al. 2001) the peak glutamine concentration was measured after ~15 minutes of administration and returned to normal after 4 hours. Importantly glutamine was given directly after the septic insult. Of course that was not achieved in our clinical trial and cannot realistically be achieved in clinical medicine with a delay from insult to presentation of hours to days. Nonetheless, evidence exists to suggest that parenteral nutrition with alanine-glutamine dipeptide at a dose of 0.5g/kg bodyweight significantly increases the serum HSP concentration after 7 days (Ziegler et al. 2005), however not as a single large bolus of intravenous glutamine.

The importance of plasma glutamine concentration was highlighted again recently in a study of > 170 critical care patients with a significantly increased mortality with plasma glutamine concentrations below 400mmol/l (Rodas et

al. 2012). Furthermore plasma glutamine concentrations above 930mmol/l were associated with increased mortality too (Rodas et al. 2012). Therefore, determination of the plasma glutamine concentration, correction of a deficiency and avoiding plasma glutamine levels above 930mmol/l rather than the overall amount of glutamine administered is of paramount importance. Routine measurements of plasma glutamine concentration are currently not feasible for practical and financial reasons (Kent and Bongers. 2011). Currently no pre-selection criteria for an "at risk" group, i.e. a group with a low plasma glutamine concentration, exist, although illness severity as well as age was suggested to correlate with low plasma glutamine concentrations (Oudemans-van Straaten et al. 2001; Novak et al. 2002)

Ample evidence exist that increased intracellular and extracellular HSP concentrations are beneficial for survival during sepsis (Le Grand and Alcock. 2012; Mc Connell et al. 2011; Aschkenasy et al. 2011). Strong evidence exist that intravenous glutamine administration correlates with intracellular and extracellular HSP concentrations ((Zhao et al. 2012; Mazloomi et al. 2011; Wischmeyer et al. 2001; Ziegler et al. 2005). It is not yet clear whether this effect is afforded by the correction of a deficiency (Oehler et al. 2002) or as a direct effect of glutamine administration (Wischmeyer P. 2006b)

5.2. Implementation for future treatments of critical ill patients

The cell culture experiments confirmed the need of a "normal" glutamine concentration during stress to generate a significant HSP response. It further supported the observation that a relative deficiency attenuated an appropriate HSP response. On the other hand, although little beneficial effect was seen at supra-maximal supplementation levels, no adverse effect was observed. Several recent large scale trials suggest a possible change of recommendation, although the trials have been criticised due to the methodology used. The results of the largest trial undertaken so far are still awaited. However, several fundamental questions remain unanswered and are unlikely to be answered.

Current evidence suggests that intravenous glutamine administration causes no significant side effects (Kent and Bongers. 2011; Rodas et al 2012) reported an increased mortality risk with plasma glutamine concentrations

below 400mmol/l and above 930mmol/l in a prospective observational trial of 174 critical care patients. No glutamine was administered during this trial. It therefore remains unclear whether glutamine concentrations above >930mmol/l following intravenous administration increases the mortality risk. The current study has suffered due to the low numbers finally recruited, which are not sufficient to show any marginal effects but have allowed power calculations to be undertaken for larger scale intervention studies . The plasma glutamine concentration was not significantly affected after one large intravenous glutamine dose suggesting the need for larger or longer supplementation levels in future studies although these need to be mindful of potentially detrimental effects of supra-maximal doses of glutamine.

5.3 Future studies

Previous data have shown that high dose glutamine administration influences the HSP 70 response in critical illness in animal sepsis models and this effect was observed in a dose dependent manner (Wischmeyer et al. 2001). Further a clinical trial showed increased serum HSP 70 after glutamine administration (Ziegler et al. 2005). The cell culture results reported in Chapter 3 do not suggest a dose dependant response of HSP 70 in muscle following glutamine administration above 'normalisation'. However the highest glutamine dose used was "only" 2.5x the normal extracellular glutamine concentration. Future studies may need to explore the question of whether higher extracellular glutamine concentrations may influence the ability of cells to respond to stress.

In an animal sepsis model a HSP response could be observed as early as one hour after glutamine supplementation (Wischmeyer et al. 2001). The clinical study reported here did not observe any effect of glutamine supplementation for up to 96 hours after treatment. Although suggested in an animal model, it is not clear whether 48hours is the best time point to investigate an HSP response in human. Future studies should therefore explore shorter time intervals. The clinical study reported here also did not show a significant change in plasma glutamine concentrations after one large intravenous glutamine dose. It is therefore likely that any future study will need to address

the problem of intravenous glutamine administration to significantly increase the plasma glutamine concentrations in any future study.

The cell culture experiments and the clinical trial suggest a correlation between glutamine concentration and intracellular HSP content, particularly the small HSPs. The relevance of this observation is not clear and will need further evaluation, potentially using transgenic mice either overexpressing or knocked out for these HSPs. Further, long term follow up trials are needed to review the role of HSPs during recovery and muscle repair.

Recent trial data of several large scale intravenous glutamine intervention trials have been disappointing suggesting no significant effect of parenteral glutamine on critical care mortality (Andrews et al. 2011; Wernerman et al. 2011; Grau et al. 2011). However, neither were the patients stratified for the risk of low plasma glutamine concentrations nor were plasma glutamine concentrations determined prior to randomisation. Any further glutamine studies need to address this knowledge deficit by

1. Identifying patients as risk from low plasma glutamine concentrations
2. Randomising patients to glutamine administration versus control with established low glutamine concentrations.

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ABSTRACT RELATED TO WORK

Poster presentation at the 2007 conference of the European Intensive Care Society, in Berlin, Germany.

Glutamine influences viability and Heat shock protein content of C_2C_{12} mouse myoblasts in culture. (No 1218)

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Studies examining the effect of glutamine supplementation in critical illness have demonstrated significant beneficial effects in animals and man although the mechanisms by which this protection occurs are not understood. We aimed to examine the effect of various glutamine concentrations on the ability of C_2C_{12} myoblasts to differentiate and its effect on Heat shock protein expression (HSP).
Methods C_2C_{12} myoblasts were raised under standard conditions. Differentiation to multinuclear myotubes was induced by replacing FCS with 2% horse serum. Cells were supplemented with glutamine at concentrations between 0 and 10mM throughout and this was replaced every other day. Photographs were taken at day 9 of differentiation. HSP content of cells was determined using western blotting as described previously (Maglara *et al*, 2003). **Results** At low levels of glutamine (0 – 0.25mM), cell survival was greatly impaired and differentiation was reduced. However HSP70 content of cells grown in media of 0.5mM and 1mM Glutamine showed an increased HSP 70 response compared with cells grown and differentiated in physiological glutamine concentrations. No effect of higher glutamine concentrations (between 2 – 10mM) on cell viability or HSC70 and HSP 70 content was evident.
Discussion Glutamine supplementation affects Heat Shock Protein (HSP) expression in various cell types. Several authors have suggested that exposure of cells to relatively high concentrations of glutamine results in increased HSP expression and an enhanced cell survival (Wischmeyer et al.1997) Skeletal muscle degeneration occurs following a number of insults and muscle repair is reliant upon activation and differentiation of stem cells or myoblasts to form mature multinucleated muscle. Transgenic studies in our laboratory have demonstrated that the ability of skeletal muscle cells to produce HSPs during stress and development is crucial to the correct maturation and functioning of these cells (McArdle et al, 2004). Our data suggests that the glutamine concentration for optimal myoblast proliferation and differentiation is ~2mM. Reduction below this value resulted in reduced cell viability and modified HSP although levels higher than physiological had little effect on cell growth and differentiation. This might suggest that reduced Glutamine concentrations itself acts as a stressful stimulus. Further reduction however renders the cell unable to respond at all.

REVIEWS RELATED TO WORK

BRITISH JOURNAL OF INTENSIVE CARE, WINTER 2011.

Exogenous glutamine: an update of the clinical evidence

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Glutamine has been shown to play an important role in a number of biochemical and immune processes. While in health it is considered a non-essential amino acid, in patients with critical illness it has been suggested that reduced endogenous production can be insufficient to meet increased demand. Exogenous glutamine supplementation has been studied extensively for more than two decades, including in several recently published large trials, but its role still remains controversial. A number of trials and meta-analyses have shown significant morbidity and mortality benefits of supplementation that have not always been replicated in other studies. There is also evidence for various effects on the immune system, antioxidant status, glucose metabolism and heat-shock protein response. This review critically appraises the current evidence regarding glutamine supplementation and the possible reasons for disparity.

Crit Care Med. 2007 Sep; 35(9 Suppl):S545-52.

Exogenous glutamine: the clinical evidence.

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Abstract

We know that critically ill patients suffering from undernutrition with a limited nutritional reserve have a poorer outcome. Furthermore, having a low body mass index has been shown to be an independent predictor of excess mortality in multiple organ failure. Therefore, nutritional support has gained increasing interest in critical illness with the hope of preventing or attenuating the effects of malnutrition. A negative nitrogen balance is the characteristic metabolic feature in critical illness, with the major protein loss derived from skeletal muscle. In particular, glutamine concentrations are rapidly reduced in plasma and muscle. Over the last 20 years or so, increasing evidence is emerging to support the use of glutamine supplementation in critical illness. Clinical trials have found a mortality and morbidity advantage with glutamine supplementation. The advantage appears to be greater the more glutamine is given and greater again when given parenterally. Various modes of action have been postulated. Glutamine seems to have an effect on the immune system, antioxidant status, glucose metabolism, and heat shock protein response. However, the benefit of exogenous glutamine on morbidity and

mortality is not universally accepted. This review critically appraises the current clinical evidence regarding glutamine supplementation in critical illness.

Curr Opin Crit Care. 2006 Apr;12(2):131-5.

Are there any real differences between enteral feed formulations used in the critically ill?

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Abstract

PURPOSE OF REVIEW: We know that adequate nutritional support is essential in the treatment of critically ill patients, because it can, if applied appropriately, improve the clinical outcome. Increasing evidence seems to suggest that malnutrition itself is a predictor of poor outcome in intensive care, and significant underfeeding during intensive care stay increases the risk of bloodstream infections. The purpose of this review is to highlight recent advances in enteral nutrition in the critically ill adult patient.

RECENT FINDINGS: Recent studies suggest that tight glycaemic control is associated with improved outcome. Enteral feeding should be encouraged, using simple feeding protocols, and started early if safe to do so. Gastric residual volumes do not correlate with the risk of aspiration, and therefore should be used with caution in feeding protocols. Conflicting evidence exists for supplementation with antioxidant and immuno-nutrition in the critically ill. Glutamine and fish oil/borage oil should be considered for burns patients and patients with adult respiratory distress syndrome, respectively.

SUMMARY: This review offers information regarding the latest developments in nutritional support via the enteral route. Further research is needed to clarify the role of enteral supplements such as antioxidants and 'immune modulating substances'.